THE EFFECTS OF ADDITIVES ON RADIATION INDUCED DAMAGE TO
DEOXYRIBONUCLEIC ACID.

thesis submitted for the degree of
Doctor of Philosophy
at the University of Leicester

by

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June 1993
This thesis is dedicated to my beloved parents Patricia and David without whose love this work would not have been attempted, and to my partner Victoria without whose love it would never have been completed.
ACKNOWLEDGEMENTS.

I am deeply indebted to my supervisor Prof. Martyn Symons FRS for his continuous support, advice and encouragement throughout the work presented here. I consider myself very lucky to have worked with someone of such great knowledge, insight and generosity.

Whilst on secondment during the summer of 1990, I spent three months working with the Biophysics group at the Cancer Research Campaign Gray Laboratory. I wish to express my sincere thanks to the group in particular to Dr. Barry Micheal, Dr. Kevin Prise, Dr. Mel Folkard and Sue Davis from whom I learned a great deal. I would also like to thank the extremely dedicated staff of the Gray and Mount Vernon Hospital for their inspiration and companionship throughout a productive and socially very enjoyable period.

I would like to thank the staff and my fellow students in the Physical Chemistry research department, in particular; Andy, Norma, Abdou, Carl, Ian, Mark, Andy, Aiden, Christine, Steve, Richie, Nigel, Graham, Heather, Saijun, Vicky, Jane and the entire Chemistry Department football team for making the lab such an enjoyable place to work. I would also like to thank the chemistry department workshop staff who built excellent equipment for the esr group often from very poor, Heath Robinson inspired sketches.

Throughout my studies I have been blessed by the great friendship of a number of people. I would like to thank my friends at Beaumont; Andy, Jason, Kev, Janine, Richard, Eric, Sue, Dan, Sue, Lucy and Kathryn for a great couple of years. I would like to thank the various members of Jahn Teller and the Distortions and The Jazzy C's in particular Greg, Danny, Andy, Andy and Nigel 'Planet Brain' Hambly, for the rock and roll.

Special thanks and 'respect is due' to the lovable rouges Warren, Matt and Mike, the '246 Boys' with whom I shared the absolute best of my years at Leicester. Special thanks and 'nough respect also to my personal shamen and guru Nic Camps.

Finally, I would like to thank my parents, Patricia and David, and my partner and co-hab Victoria without to whose love this work would not have been completed.
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1.1. Preview.

The effects of exposing living systems to radiation have been subject to intense research for many years. The results of such exposure are complex and of serious consequence to whole organisms and to individual cells. Exposure may result in cell death, mutagenesis and carcinogenesis.

Human beings are not only exposed to natural sources of radiation but also to radiation from nuclear weapons testing and nuclear industrial accidents such as the tragedy in Chernobyl. The long term effects of such exposure are still not entirely known. It seems likely that nuclear weapons will continue to proliferate and that nuclear power will play an increasing rôle as an energy source as other fuel stocks are exhausted. To ensure the future safety of workers in these industries and of society in general, a full understanding of the effects of radiation on living systems is clearly important.

The use of radiation in radio-therapy is a tremendously important method of treating certain types of cancer. Research into the mechanisms of radiation damage to the cell and the repair processes that may follow is vital to the future development of this life saving technique.

In light of these factors, the study of the effects of radiation on living systems is not simply of academic interest.

The structural integrity of cellular deoxyribonucleic acid (DNA) is vital for normal cell function. Whilst radiation induced damage to the cell is believed to be harmful in
general, it is now believed that radiation induced damage to the DNA leads to such phenomena as cell death, mutagenesis and carcinogenesis.

The studies presented here centre on both the effects of ionizing radiation on DNA and also the radioprotective and radiosensitising effects additive compounds may have upon these. The ultimate aim is to contribute to the development of drugs which may either provide protection to living systems from the harmful effects of radiation or selectively enhance cancer radio-therapy techniques.

1.2. An overview of the effects of ionizing radiation on living systems.

The effects of exposing living systems to ionizing radiation are extremely complex and may be expressed over a wide time scale (Figure 1–1). Ionizing radiation may be defined as any radiation consisting of either directly or indirectly ionizing particles or electromagnetic quanta. Exposure of biological systems to such radiation results in the physical processes of energy deposition (timescales of $10^{-18}$ to $10^{-12}$s). Initially energy deposition from ionizing radiation results in primary ionizations. The products of these ionizations, such as emitted electrons, may in turn cause further or secondary ionizations. The complex nature of the fundamental processes governing energy deposition and dose, and variations in these with radiation quality for a wide range of ionizing radiations is discussed by Keifer and Faharaziz and Rodgers. Computer models of ionization 'tracks' have been used to study the nature and variations in the density of ionization for DNA models and several radiation qualities. Such areas are still subject to intense research.
The products of ionization undergo chemical reaction via radical chemistry resulting in chemical changes to the DNA (timescales of $10^{-8}$s to 10ms). Such changes lead to biological responses within the cell. Radiation induced damage repair may occur in two ways. Firstly 'fast' (timescales of 10ms) chemical repair of DNA radical species by for example free thiol groups such as that of glutathione$^{[4-5]}$. However the mechanisms and nature of all possible 'fast' chemical repair responses of the cell are not completely understood. Secondly, enzymic repair of chemical alterations to the DNA structure (described below) also occur (timescales of minutes–hours).

Exhaustion of these repair processes by overwhelming doses of radiation may lead to cell death (timescales of hours to days), where the ability to produce viable progeny by mitosis is lost (this may not be expressed for several cell divisions). However the distinction must be made that a single damage event can result in cell death even when repair mechanism exist and function within the cell. The incorrect repair of DNA lesions or chemical alterations may also be expressed as cell death, but also may result in mutagenesis or carcinogenesis (timescale of years).

1.3. The chemical structure and organisation of DNA within the cell.

An understanding of the structure of DNA, not only of the isolated molecule but also of its quaternary structure and organisation within the cell is essential to a full understanding of the effects of radiation upon it and hence the consequences to cell function. As initially proposed by Watson and Crick, DNA consists of two Figure
1–1. A schematic representation of the processes which occur following exposure of the cell to ionizing radiation.

anti-parallel polynucleotide chains coiled in a duplex about a common axis. The backbone of each strand consists of deoxyribose sugar residues linked by phosphodiester bridges between the 3'-carbon atom of one sugar residue and the
5'-carbon atom of the next (Figure 1-2i). Each deoxyribose residue is bonded to one of four bases; guanine[G], adenine[A] (purines), thymine[T] and cytosine[C] (pyrimidines) at the 1'-carbon atom of the deoxyribose residue via an N-glycosylic bond. The bases are complementary between the two polynucleotide chains; the two strands are held together by hydrogen bonding between the complementary guanine and cytosine, and adenine and thymidine base residues. The structure and hydrogen bonding of these base pairs is illustrated below (Figure 1-2ii).

Figure 1-2. i) The primary structure of DNA, illustrating the phosphodiester chain of the sugar phosphate backbone.
ii) The structure and hydrogen bonding of the DNA base pairs.

The resulting duplex has its base pairs stacked in parallel forming the core of the helix, the strands being anti-parallel as the 5'-P terminus of each strand is adjacent to the 3'-OH terminus of the complementary strand.

DNA has been shown to exist in several helical forms termed the A, B, C and D forms. The Z form has also been determined in which the sugar–phosphate backbone
exists as a left-handed helix. The A, C and D forms predominate in low ionic strength or at specific relative humidities; the B form is thought to be the native form[6].

In the cell, the DNA's higher order structure is highly organised since in mammalian cells each chromosome consists of approximately $8 \times 10^8$ base pairs so these huge molecules must not simply be accommodated within the cell nucleus but must be arranged in such a way as to allow the genetic information of any section to be readily copied during transcription. Within the cell the DNA exists as complexes with protein molecules to give chromatin.

DNA is closely associated with many types of DNA binding protein which may be subdivided into histone and non-histone chromosomal proteins. Whilst non-histone proteins may influence DNA folding in specific regions, it is DNA binding to the histone proteins which induces the bulk folding of the DNA into chromatin. Histone proteins have relatively small molecular weights and contain a high proportion of the positively charged lysine and arginine amino acid residues which facilitate electrostatic binding to the DNA. Five types of histone protein are involved in chromatin. The histones H2A, H2B, H3 and H4 are bound with DNA into nucleosomes which are the fundamental repeating units of chromatin. The DNA is bound to the outer surfaces of a histone complex which contains a pair of each of the histones. Each nucleosome unit contains approximately 150 DNA base pairs. Nucleosomes are linked continuously to each other via short sections of unbound DNA of around 60 base pair units which are termed 'linker' DNA. The remaining histone protein H1 is believed to bind adjacent nucleosomes together, further compacting the DNA molecule. DNA–protein complexes have been reviewed by Champoux[7] and Von Hippel[8].
1.4. Initial interaction of gamma–radiation and matter.

Ionizing radiation may be defined as any radiation consisting of directly, or indirectly ionizing particles or photons. Whilst high energy photons interact sparsely with matter, occasional interactions of these photons with an atomic nucleus or an orbital electron results in an ionization event. The principle ionizing radiation sources used for the studies presented here were $^{60}$Co 1.17 and 1.31 MeV gamma–rays. The interaction of a photon of gamma–radiation with an orbital electron is thought to result in the majority of the photon's energy being given to the electron, causing the electron to be ejected from the atom. These high energy electrons may give rise to further ionizations and excitations by collision with other orbital electrons. Such emitted electrons are responsible for the majority of damage induction with indirectly ionizing radiations.

1.5. The radiation chemistry of DNA.

In order to discuss the molecular level effects of ionizing radiation, a distinction is usually drawn between the direct and indirect effects of radiation upon DNA.

The simplest view is that 'direct' effects are due to the absorption of radiation by the DNA molecule itself. 'Indirect' effects are due to the reaction of the DNA with diffusible radical products formed by the radiolysis of the DNA's solvent environment. Both indirect and direct processes may result in damage to the DNA structure. (As shown below, this simple definition leaves open an important method of damage and is therefore somewhat ambiguous.)
The radiation chemistry of DNA itself is complex, since ionizing radiation is not selectively absorbed by DNA alone. In isolated DNA systems conditions may be modified to allow the study of specific mechanisms of DNA damage by ionizing radiation. Within the cell, the environment of the DNA is dramatically more complex, however there is a large and growing body of evidence and it is now generally believed that DNA double strand breaks are the important lesion leading to most cellular effects and to cell death.

Radiation induced chemical alterations to DNA structure may take several forms;

i) **Single Strand Breaks**, ssb.

These are scissions in the sugar phosphate backbone of one strand of the duplex, and may be quantified in a number ways; the mechanisms by which such lesions may occur is discussed below.

ii) **Double Strand Breaks**, dsb.

These may be the result of a single energy deposition or the interaction of two single strand breaks formed in close proximity to each other. The contribution of these two processes are reflected by dose response curves. Frey and Hagen\cite{9} demonstrated that direct formation of dsb in dilute aqueous solution is of very minor importance, the formation of dsb being quadratic and a result of OH derived ssb. However within the cell, for doses of above 10Gy, the dose response is linear indicating the direct formation of dsb is of far greater importance, *Radford* demonstrated that dsb may be linearly related to lethal lesions strongly supporting the belief that dsb are the important lesions leading to cell death\cite{10}. This linear relationship has also been demonstrated by *Prise et al*\cite{11}.
Both single and double strand break yields have been used in the studies reported here.

iii) **Base Damage and Base Loss.**

Exposure of aqueous DNA to ionizing radiation results in a dose dependent reduction in optical absorbance at 265nm. This has been attributed to base damage and base loss.

iv) **Intramolecular and DNA-protein cross links.**

Intramolecular DNA cross link formation results in large changes in DNA melting profiles and may interfere with DNA transcription should they not be correctly repaired. DNA-protein cross links may also occur since DNA within the cell is closely associated with many proteins. Such cross links have been studied by Oleinick\textsuperscript{[12&13]}.

It must be stressed that these types of DNA damage are not mutually exclusive, in fact often quite the contrary, for example base loss or damage may induce local denaturing and strand breaks are often accompanied by base loss.

1.6. **The indirect effects of ionizing radiation.**

Water accounts for between 80 to 90\% of the total weight of the cell, thus for indiscriminately ionizing radiation such as the gamma-radiation used in these studies, water radiolysis may also account for at least 40\% of the total ionization events within the cell. The highly mobile and reactive water radiolysis products may react with the cell's biomolecules including the DNA. Thus the indirect mechanism must be
significant. An understanding of the indirect component of DNA damage can only be
gained with a full understanding of the radiation chemistry of water.

In the absence of oxygen, the absorption of ionizing radiation by a water molecule
results either in excitation or ionization (Reaction 1). The excited molecules undergo
homolytic splitting yielding hydrogen atoms, H , and hydroxyl radicals, OH (Reaction
2).

\[ \text{H}_2\text{O} \rightarrow \text{H}_2\text{O}^* + e^- \quad (1) \]

\[ \text{H}_2\text{O} \rightarrow \text{H}_2\text{O}^* \rightarrow \text{H} + \text{OH} \quad (2) \]

Thus the initial deposition of energy results in the formation of H, OH, H\text{O}^* and
e\text{. The lifetime of the H\text{O}^* species is extremely short (of the order 10^{-14}s); further
reaction with water molecules results in the formation of hydronium ions, H\text{3O}^\text{+}
and
further hydroxyl radicals (Reaction 3), although a small number of electron-transfer
processes may occur prior to this (Reaction 4).

\[ \text{H}_2\text{O}^* + \text{H}_2\text{O} \rightarrow \text{H}_3\text{O}^\text{+} + \text{OH} \quad (3) \]

\[ \text{H}_2\text{O}^* + \text{H}_2\text{O} \rightarrow \text{H}_2\text{O} + \text{H}_2\text{O}^\text{+} \quad (4) \]

The emitted electrons may cause further excitations or ionizations or become
hydrated to form aqueous electrons, e\text{aq}^\text{+}, which are less reactive. Such energy
dissipation results in the formation of small localised regions containing high
concentrations of ions and free radicals known as spurs, blob or track ends. The concentration of these reactive units rapidly diminishes due to dispersive diffusion, during which further reactions may occur. Solvated electrons may react in a number of ways (Reactions 4-8):

\[
e_{\text{aq}}^- + H_3O^+ \rightarrow H^+ + H_2O \quad (5)
\]

\[
e_{\text{aq}}^- + H + H_2O \rightarrow H_2 + OH^- \quad (6)
\]

\[
2e_{\text{aq}}^- + 2H_2O \rightarrow H_2 + 2OH^- \quad (7)
\]

\[
e_{\text{aq}}^- + H_2O_2 \rightarrow OH^- + OH \quad (8)
\]

Some recombination reactions are also possible (Reactions 9-11):

\[
OH^- + OH \rightarrow H_2O_2 \quad (9)
\]

\[
H + H \rightarrow H_2 \quad (10)
\]

\[
H + OH \rightarrow H_2O \quad (11)
\]

Thus overall, energy deposition within the solvent results in the formation of hydrogen atoms, solvated electrons and hydroxyl radicals. The probabilities of these reactions is largely dependent on the local concentrations of the reacting species.
within the individual tracks or spurs. It must be stressed that interaction with solutes may occur only if they are themselves within the spurs. However the reactive hydrolysis products H, OH and e\textsubscript{aq} are able to escape these spurs or tracks, and may react with solute molecules located in close proximity. Thus cellular DNA damage may only occur if these spurs or tracks are in relatively close proximity to the DNA. The likelihood of such DNA damage is discussed below.

In the cell under normal circumstances, oxygen is present. The radiobiological effects of oxygen are profound and complex, and are not discussed here. However the reaction of molecular oxygen with solvated electrons or hydrogen atoms yields further diffusible and reactive products which may subsequently cause DNA damage (Reactions 12–14):

\begin{align*}
O_2 + H & \longrightarrow HO_2 \quad (12) \\
O_2 + e_{aq} & \longrightarrow O_2^- \quad (13) \\
HO_2 + HO_2 & \longrightarrow H_2O_2 + O_2 \quad (14)
\end{align*}

Studies using dilute aqueous DNA irradiated at room temperature have demonstrated that OH radicals are the principal cause of DNA damage. Extensive studies using model compounds have shown that OH radicals attack DNA in a relatively unselective way.

Hydrogen atom abstraction from the sugar residues is believed to account for approximately 20% of OH radical attack on DNA; the most significant and well
characterised mechanism for this being H-atom abstraction from the 4' C-atom of the deoxyribose residue. The resulting sugar radical may undergo β-elimination resulting in a scission of the sugar-phosphate backbone, commonly termed as a strand break (Figure 1-3).
Figure 1–3. The $\beta$-elimination mechanism by which 4' C-atom centred sugar radicals may yield strand breaks.

Base–OH radical adduct formation has also been shown to lead to strand breakage and is believed to account for the majority of OH radical damage to DNA. Intramolecular H-atom transfer from the sugar residue to the base–OH radical centre with the subsequent loss of $H_2O$ again results in sugar radical formation. The resulting sugar radical may also undergo $\beta$-elimination as described above.

DNA damage may also result from reaction with solvated electrons ($e_{aq}^-$), which may form radical cations centred on the pyrimidine base residues. Similar reactions to those described below for the direct effects may occur, again resulting in strand
breakage. The radiation chemistry of water is discussed in detail by Faharaziz and Rogers[^2].
1.7. The direct effects of ionizing radiation.

Direct irradiation results in absorption of energy by DNA and yields electron-loss and electron-gain centres within the molecule itself (DNA → DNA$^+$ + DNA$^-$). Partial π-orbital overlap within the DNA's central base stack is believed to allow both 'hole' and electron migration by charge transfer. The precise mechanism of this process is not fully understood. Various mechanisms for charge transfer processes are discussed by Baverstock and Cundall$^{14}$. Such electron-loss and electron-gain centres may be studied by electron spin resonance (esr) spectroscopy.

Early esr studies of irradiated dry oriented DNA fibres were carried out by Graslund et al$^{15,16}$. Studies of frozen aqueous DNA samples irradiated at low temperatures (usually 77K) have been carried out by Hüttermann et al$^{17}$, Gregoli et al$^{18}$ and Symons et al$^{19}$.

Frozen aqueous DNA samples are used in the studies presented here and it is necessary to consider the nature of this system in order to understand the results. During freezing, ice crystallite formation occurs with the exclusion of solute molecules. These crystallites bind most of the water molecules of the system. In studies using HOD–D$_2$O, ice crystallite water molecules are observed as a narrow band at ca. 3280 cm$^{-1}$ superimposed over a weak broad band assigned to solvating water. The solvating water has a glass like structure. The water molecules strongly interact with both the (RO)$_2$PO$_2^-$ residues of the DNA and their sodium counter ions, and the base residues are also strongly solvated. Under these conditions irradiation results in direct irradiation of DNA which is phase separated from the bulk ice phase. Hydroxyl radicals formed within the ice phase are trapped and may not cause DNA
damage. Annealing of samples to 130K results in the irreversible loss of these radicals; this corresponds with the loss of OH radicals in pure ice. The product \( \text{H}_2\text{O}_2 \) remains trapped within the ice phase until melting. The great advantage of this system is that additive compounds are incorporated into the DNA solvating regions and their effects upon direct DNA damage can be studied. Studies incorporating hydrogen peroxide into the DNA solvating region have demonstrated that DNA damage from OH radicals formed within the DNA solvating region does occur, to give primarily, sugar centred radicals\(^{[20]}\). Such sugar radicals are not observed in pure aqueous samples, supporting the premise that the observed OH radicals are trapped within the ice crystallites and do not enter the DNA phase.

Early esr studies characterised the primary electron loss and electron gain centres as being guanine cations \([\text{G}^+]\) and thymine anions \([\text{T}^-]\)\(^{[18,21]}\). The electron loss centre is still thought to be localised on guanine \([\text{G}^+]\) (Figure 1–4i.), however recent publications have suggested that electron capture, whilst being largely centred on the pyrimidine bases, is not a selective process and that cytosine \([\text{C}^-]\) (Figure 1–4ii.) and thymine \([\text{T}^-]\) (Figure 1–4iii.) radical anions are formed\(^{[22-24]}\). Although a strong case for electron capture by thymine residues has been put forward\(^{[25]}\), the site of electron localisation remains unresolved.
Figure 1-4.
i) The structure and first derivative X-band esr spectrum of G⁺.
ii) The structure C⁻.
iii) The structure and first derivative X-band esr spectrum of T⁻.
iv) The first derivative X-band esr spectrum of RO₂.

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The primary DNA radical ions G⁺ and C⁻/T⁻ are stable at 77K. Their esr spectra are seen as a composite of the G⁺ singlet (25 G) and the indistinguishable doublets (17 G) of C⁻/T⁻. In addition to these features, the 1st derivative spectrum at 77K also contains the parallel and perpendicular features of OH radicals which are trapped within the ice crystallites (Figure 1-5). Annealing to 130K results in the irreversible loss of hydroxyl radicals (the product H₂O₂ remaining trapped in the ice crystallites until thawing), leaving equal yields of G⁺ and pyrimidine anions C⁻/T⁻. On further annealing, T⁻ is readily protonated from the solvent at the 6' C-atom, forming TH⁻ (Figure 1–6i.). TH gives rise to an unambiguously assigned octet esr spectrum (Figure1–6ii.) with the corresponding loss of the doublet feature from the central DNA composite feature. TH continues to increase in intensity until a maximum at ca. 210K. A plot of the intensities of these radicals versus temperature demonstrates their behaviour on annealing (Figure 1–7.).

The presence of oxygen results in the formation of the uncharacterised RO₂ species by reaction at the anion centre which results in reduction in TH yield on annealing (Figure 1–4iv.).

The mechanism by which these radical centres give rise to strand breakage has not been revealed by esr studies. DNA strand breaks may be quantified in supercoiled plasmid DNA. After exposure to ionizing radiation single strand breaks result in the relaxation of supercoiling in the plasmid pBR 322, resulting in an open circular form. Double strand breaks result in relaxation into a linear form. The three forms of the this DNA may be separated by gel electrophoresis and from the relative intensities of
the resulting bands, the yield of these two types of lesions may be quantified. This is represented schematically in figure 1–8.
Figure 1–5. First-derivative X-band esr spectrum of deoxygenated aqueous DNA after exposure to $^{60}$Co gamma-rays at 77K, showing features assigned to OH radicals and composite features of DNA primary radicals, guanine cations [$G^+$] and pyrimidine anions [$C^-/T^-$].

Figure 1–6. The structure and first derivative esr spectrum of TH radicals containing the characteristic octet feature.
Figure 1–7. A plot of the intensities of DNA primary and secondary radical features versus temperature.

Conditions of direct damage may be produced by irradiation at 77K. Such studies have demonstrated that ca. 25% of the DNA primary radicals formed under direct damage conditions give rise to strand breakage\textsuperscript{[26,27]}.

It has been suggested that strand breakage occurs via neutral sugar residue centred radicals, and that this is likely to occur as a result of hydrogen atom transfer from the sugar residues positioned close to the base centred radicals\textsuperscript{[28,29]}. In fact several such sugar C–H units are 'poised' close to base radical centres, and for TH this seems a likely mechanism to form sugar centred radicals which may in turn decay to yield strand breaks (Figure 1–9). This mechanism has been further supported by the report that TH\textsubscript{2} residues are a major product of aqueous DNA irradiation at 77K\textsuperscript{[30]}. 

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The mechanism by which G' centres may yield strand breaks is less clear, reaction with water molecules may give rise to G OH or G centres which may in turn yield sugar radicals by the same route as TH above.

Figure 1-8. Schematic representation intact supercoiled, open circular and linear forms of pBR 322 plasmid DNA.

1.8. The significance of indirect and direct components of total DNA damage within the cell.

Experiments with dilute aqueous DNA demonstrate that the majority of initial energy deposition occurs within the solvent and that DNA damage a largely due to chemical attack by solvent radiolysis products. It has been shown that hydroxyl radical [OH ] induced DNA damage dominates total DNA damage in dilute aqueous
systems irradiated at room temperature. However the DNA environment within the cell is radically different and direct DNA damage by ionizing radiation is of much greater significance.

Whilst the bulk of the cell does chiefly consist of water, most of this is confined to the cytoplasm. Highly reactive water radiolysis products formed within the cytoplasm cannot reasonably be considered to cause DNA damage.

Within the nucleus, DNA is densely compacted with histone proteins into chromatin with the exclusion of much water, thus the effective solvent target is greatly reduced compared with that of aqueous isolated DNA systems. Hydroxyl radicals react relatively unselectively and, given the high concentration of many other biomolecules such as ribonucleic acids [RNA's] and non-histone proteins within the nucleus, only limited OH radical damage to DNA may be expected. Consequently solvent radiolysis product derived DNA damage, predominantly due to OH radicals, is less likely.

Within the nuclear environment of DNA, the high abundance of polar macromolecules (such as DNA itself) results in perturbations in the water structure. The perturbed molecules form solvating regions in which the primary water radiolysis products, which are non-solvated or \textit{dry} (\(\text{H}_2\text{O} \rightarrow \text{H}_2\text{O}^{\text{dry}} + e^{-}\)), are transferred to the DNA by charge transfer before the more long-lived molecular water radiolysis products are formed. Radiation studies using hydroxyl radical scavengers in mammalian cells\textsuperscript{[1]} have shown significant DNA damage despite the efficient scavenging of OH radicals, which must be due at least in part to direct damage.
Figure 1–9. Local structure of a TH radical and surrounding DNA, showing how a 'poised' hydrogen atom may be transferred to form a sugar radical.
References for Chapter 1.


CHAPTER 2. EXPERIMENTAL TECHNIQUES AND PROCEDURES.

2.1. Introduction.

The aim of this chapter is to describe in detail the experimental techniques and procedures employed in the studies of ionizing radiation damage to DNA presented in this thesis.

Extensive use was made of electron spin resonance spectroscopy (esr) to study the influence of several compounds upon the nature and mechanism of gamma–radiation damage to DNA in frozen aqueous systems.

Neutral (pH 9.6) filter elution techniques based on those developed by Kohn et al\textsuperscript{(1)} were also used to monitor low linear energy transfer (LET) radiation induced DNA double strand breaks in studies of the effects of novel polyammonium cation nitroimidazole compounds.

2.2. Electron spin resonance spectroscopy.

2.2.a. Preparation of Samples.

Typically solutions of 25 or 50 mg ml\textsuperscript{-1} of calf thymus DNA sodium salt (BDH) in water were prepared giving final DNA concentrations of 0.075 and 0.15M DNA base pairs respectively (average molecular weight per base pair in calf thymus DNA being 660.4g). 0.15M samples were only used when low additive to DNA base pair ratios
were required. Additive compounds were included in the initial solutions prior to DNA being added.

In order to produce more clearly defined spectra of primary DNA and additive derived radicals, samples were also prepared in D₂O (BDH). However these samples give less information of the fate of DNA primary radicals on annealing. It must be stressed that the two solvents must be used in tandem to obtain the fullest understanding of radiation induced radical mechanisms; H₂O provided information on subsequent reactions of primary radicals and D₂O provided information on primary radical quantification.

These gel like solutions were the mixed thoroughly and then incubated at 4°C for 48 hours. After this period the solutions were again thoroughly mixed to ensure complete homogeneity.

The presence of molecular oxygen in samples results in the formation of the uncharacterised oxygen adduct radical ROO on annealing of the irradiated sample. (The effects of oxygen in these systems is discussed by Symons et al[2]) This obviously interferes with the study of DNA derived radicals and the influence of additives on their subsequent decay. To avoid RO₂ formation, each sample was degassed by passing nitrogen gas (which has been scrubbed free of even trace amounts of oxygen) through each sample solution whilst under a nitrogen atmosphere prior to freezing.
2.2.b. Oxygen removal from nitrogen.

Initially, nitrogen gas ('oxygen free' grade, BOC) was passed through alkali pyrogallol solutions to remove trace amounts of oxygen prior to being passed through the sample. For comparison two identical samples of aqueous DNA were degassed using pyrogallol scrubbed nitrogen and argon respectively, these were then frozen, gamma-irradiated and their esr spectra recorded as described below. Whilst neither sample after annealing contained features assigned to RO₂, the TH yield in the pyrogallol/nitrogen degassed sample was slightly diminished suggesting the presence of trace amounts of oxygen, reducing TH yield.

Two more efficient methods of oxygen removal were adopted both of which were based on transition metal redox cycling. In both cases the nitrogen gas was passed via glass sinters through two sealed flasks in sequence containing solutions of aquated Cr" or V" ions which were readily oxidized by any molecular oxygen present in the gas. The gas was then passed through D₂O to remove any aerosols which might otherwise contaminate the samples. This also had the effect of saturating the gas and hence prevent sample dehydration. The redox reactions involved in each system are described below.

2.2.c. Chromium II/III scrubber.

Blue aquated Chromium II ions, [Cr(H₂O)₆]²⁺, were prepared by the reduction of green hexa aquo Chromium III ions, [Cr(H₂O)₆]³⁺, with zinc amalgamated with mercury (Zn/Hg) in strongly acidic conditions³⁴. The resulting blue solution is
producing a deep violet solution which again readily scavenged the trace amounts of oxygen present in the nitrogen gas\(^8\) (Scheme 2–2). In this case the loss of the intense violet colour indicating solution exhaustion.

Scheme 2–2. A schematic representation of the oxidation of \([\text{V(H}_2\text{O)}_6]\)^2+ ions by molecular oxygen and the subsequent reduction by Zn/Hg amalgam.

\[
2[\text{V(H}_2\text{O)}_6]^2+ + \text{O}_2 \rightarrow 2[\text{V(H}_2\text{O)}_6]^3+ \quad \text{Blue}
\]

Once degassed with respect to oxygen, the homogeneous sample gels were carefully formed in liquid nitrogen into uniform cylindrical pellets, 30mm in length and 4mm in diameter.

All samples were gamma-irradiated in a Vickers \(^{60}\)Co source for 4 hours. The dose rate of 20 Gy min\(^{-1}\) was measured using Fricke dosimetry\(^7\).

2.2.e. esr measurements.

All spectra were recorded at 77K. Each sample pellet was placed in a quartz finger dewar filled with liquid nitrogen. The dewar was then inserted directly into the spectrometer cavity.
Great care was taken to ensure that the dewar was placed in the same position for each sample, that the sample completely filled the spectrometer cavity vertically and that the sample was always orientated in the same way (the 'top' of each sample being marked). Since the samples are of identical uniform dimension, their distortion of the magnetic field in the cavity is close to identical. The response of the esr spectrometer cavity varies vertically and is at a maximum sensitivity in the centre. The samples were positioned so as to completely fill the 'detected' cavity, ensuring the minimum error due to this non linearity.

The total error due to sample positioning was assessed by recording the esr spectrum of an irradiated 25mg ml	extsuperscript{−1} DNA sample at 77K after it had been annealed to 130K ten times. The sample being removed from and replaced into the dewar and cavity between each recording. The total variation in the peak height of the central feature proved to be less than 5 per cent total, significantly less than any augmented or diminished yield relative to controls reported in this thesis.

The first derivative X–band esr spectra were recorded using either a Varian E109 or a Jeol X–101 spectrometer, both spectrometers had 100 kHz magnetic field modulation and were interfaced to Acorn Archemeides A3000 microcomputers. Spectra were recorded by computer accumulation using software written and supported by Mr J.A. Brivati of this department. The software allowed rapid spectra storage, subtractions and the double integration of spectra. Multiple accumulation allowed great improvement in signal to noise ratios where sample radical populations were low, or when high amplifier gains were required. g–tensor values were measured relative to a retractable manganese g–marker fitted to the spectrometer.
Sample annealing was carried out by placing the sample pellets into pre-cooled
glass tubes under liquid nitrogen. The nitrogen was then decanted off and the tubes
placed in one of twelve accurately drilled 50mm deep holes in the top end of a solid
brass cylinder (60mm diameter and 300mm length) which was maintained at the
required temperature (±0.5K). The temperature of the block was monitored by a
platinum resistance thermometer positioned at the same depth as the samples. Brass
was used as the material to give high thermal conductance and hence minimal
temperature gradient between the thermometer position and the sample position. Up
to twelve samples could be simultaneously annealed in this way. After annealing for 8
minutes the samples were again placed under liquid nitrogen to quench any
proceeding reactions.

2.3. Quantification of radiation induced DNA double strand breaks (DSBs) by
neutral (pH 9.6) filter elution.

2.3.a. Cell Culture.

A Chinese hamster V79-379a lung fibroblast cell line, a gift from Dr. Kevin Prise
of the Cancer Research Campaign Gray Laboratory (PO Box 100, Mount Vernon
Hospital, Northwood, Middlesex. HA6 2JR), was maintained in log phase growth
either on flat plates or in suspension. Eagle's minimal essential medium, MEM (ICN
Flow) was used in both cases supplemented with 7.5% (v/v) foetal calf serum for
suspension cultures and 10% (v/v) foetal calf serum for cultures where cells were
grown attached to flat plates. The medium in both cases was supplied supplemented
with penicillin and streptomycin. The cultures were maintained under an Air(95%) CO₂(5%) (BOC) atmosphere.

2.3.b. Sample preparation and irradiation.

Exponentially growing cells were trypsinised and counted after re-suspension in suspension modified MEM; this medium being used to prevent cell attachment to the walls of the irradiation tubes. The cells were aliquoted out into sterile 2ml screw cap cryotubes (Flow) in two main fractions, their density having been adjusted to give 2 x 10⁶ cells per aliquot; the first fraction being the control, the second dosed with the required concentration of drug compound.

Since the polyammonium cationic nitroimidazole compounds were supplied in the hydrochloride form, care was taken to ensure the dosed fraction did not experience any pH shock. This was done by careful pH measurement and adjustment of the dosed media prior to the addition of the V79 cells.

These aliquots were then cooled on ice for 40 minutes. Hypoxic conditions were generated by placing the cooled aliquots again on ice in a continuously purged nitrogen(95%):CO₂(5%) (BOC) atmosphere for 90 minutes prior to irradiation.

Gamma-irradiation was carried out in a Vickers 'Vickrad' ⁶⁰Co gamma source. Each sample aliquot was enclosed in a pre-cooled lead sleeve to reduce the dose rate. The temperature of the lead sleeve was maintained at 0°C for each irradiation to minimise enzymic repair processes within the irradiated cells. The dose rate of 0.0998 Gy s⁻¹ was measured by Fricke dosimetry.
Once irradiated the sample aliquots were again placed on ice prior to lysis and elution.

2.3.c. Filter Elution.

Filter elution was carried out using the Swinnex funnel column system based on that described by Kohn et al\(^1\). Swinnex syringe barrels were connected to aluminium foil covered 25mm swinnex filter holders (Millipore, UK) which contained 2.0 \(\mu\)m polycarbonate filters (Millipore). Stop–cock valves (a gift from the Biophysics Group CRC Gray Laboratory) were connected to the bottom of this assembly to control the flow of fluids through the filter holder (Figure 2-1).

Ice cold phosphate buffered saline solution (PBS, Aldrich tablet form) was used to charge the filter assembly prior to use; the solution being slowly injected from the bottom of the assembly via the stopcock valves whilst the filter holders were continually agitated to ensure no air bubbles were trapped in the filter holders. The combination of trapped bubbles and fluid flow during elution has been shown to cause DNA shearing during elution\(^1\) and was thus avoided in this way. Once the filter holders were full the syringe barrels were topped up with further ice cold PBS to 20ml, and the whole assemblies kept refrigerated until use.

Irradiated cells were added to the PBS in the syringe barrels and the sample tube rinsed into this with a further 5ml of ice cold PBS. The solutions were then allowed to drip through the assemblies until the solution meniscus reached the bottom waist of the syringe barrel (as marked in Figure 2–1, the fluid levels were never allowed
below this point). The barrels were topped up again with a further 10ml of PBS and the process repeated to ensure the cells were deposited on the filters.

![Diagram of the Swinnex funnel filter holder assembly. The minimum fluid level is indicated.](image)

Figure 2-1. Diagram of the *Swinnex funnel* filter holder assembly. The minimum fluid level is indicated.

1 ml of lysis solution (as supplied at 0.06% (v/v) Sarkosyl NL-30 in 0.04M Na₄EDTA (tetrasodium salt) solution plus 1mgml⁻¹ Proteinase K added immediately
prior to use) was then run onto each filter in the same way. Once the flow was stopped each column was incubated for 1 hour at room temperature.

40 ml of pH 9.6 eluting solution (1.2% (v/v) tetrapropylammonium hydroxide (Pr₄NOH) in 0.02M H₄EDTA (free acid) solution, pH 9.6) was then added to the syringe barrels and the stopcock taps connected to 0.89mm plastic peristaltic tubing via 19 gauge stainless steel hypodermic needles (Richardsons, Leicester). Stainless steel needles are required because aluminium needles were found to react with the caustic eluting solution. The tubing was then passed through a slaved reversible slow speed peristaltic pump (constructed in the workshops of this department) and connected to a 10 x 10 array automated fraction collector designed by Dr Yvette Goward (again constructed in the department workshops).

Once connected, the stopcock valves were opened and each column was pumped to waste for 30 minutes at 0.035ml min⁻¹. For each column, ten fractions were then collected each over a 90 minute period into 4ml sample cups (Richardsons) at the same pump rate. The fraction collector was timed to switch off the slaved pump after this was completed.

2.3.d. DNA quantification of the eluted fractions.

The quantitation of the DNA of each fraction was carried out by fluorimetric analysis using Hoechst 33258 dye,

2'-((4-hydroxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5'-bi-1H-benzimidazole trihydrochloride pentahydrate.

43
The suitability of the dye for this type of quantitative determination has been demonstrated by Cesarone et al, and a discussion of the advantages this offers such as DNA specificity has also been published. For these analyses, it was necessary to buffer the dye to neutralise the high pH of the eluting solution. It has been shown that the Hoechst 33258 fluorometric assay produces comparable results to those using [14C] thymidine–labelled cells, and a detailed comparison of the two techniques has been published by Murray et al. The automated method described here was developed from that described by Stout and Becker.

The eluted volume of each fraction was recorded and the sample cups placed onto the carousel of a Chemlab CS 40–80 auto sampler, set to sample for 70s and wash for 90s. The sample aliquot of each fraction was then pumped using a Chemlab CPP 15 peristaltic pump through a glass mixing coil where it was thoroughly mixed with pre-buffered Hoechst 33258 dye solution (1mM sodium citrate in 0.04M KH2PO4 plus Hoechst 33258 dye immediately prior to use) and air, yielding a final dye concentration of 5 x 10^-8 M, which was found to give the most linear response to DNA concentration.

Once mixed the buffered DNA–dye solution was passed through a de-bubbler and through the quartz flow cell of a Shimadzu RF–540 spectrofluorophotometer fitted with a Shimadzu DR–3 data recorder and a Shimadzu C–R6A Chromatopac integrator. A flow diagram of the analysis system is given below (Figure 2–2). The dye was excited at 350nm wavelength and the emission recorded at 90° to the excitation source at 475nm wavelength.

The Hoechst 33258 dye–Spectrofluorophotometer response was calibrated for each full analysis run by preceding and following complete fraction analysis with a series
of ten DNA standards, increasing in increments of 0.1 \( \mu g \text{ ml}^{-1} \) from 0.1 to 1.0 \( \mu g \text{ ml}^{-1} \).

Each of the series of ten sample fractions for each column were separated by three cups; the first and the third containing eluting solution the second containing a standard 0.5 \( \mu g \text{ ml}^{-1} \) DNA solution to allow any variation in Hoechst 33258

---

**Figure 2-2.** A flow diagram of the fraction analysis system.

dye–Spectrofluorophotometer response with time to be monitored and subsequently corrected for.
The DNA retained on the filters and in the peristaltic pump tubing was collected for quantitation by removing the syringe barrels, inverting the filter holder assemblies and then back flushing each column with a further 4ml of eluting solution into 25ml autoclavable screw cap bottles (Harris). The stopcock taps were then removed and the filter holders carefully unscrewed. The filters were removed and added to the backflushed volume using tweezers. Care was taken not to handle the filters or the internal surfaces of the filter holders to prevent nuclease contamination. The filter holders were then washed twice with two 5ml volumes of eluting solution, these washes were also added to the backflushed volume.

To ensure dissolution of all the DNA from the filters these solutions were then autoclaved for 20 minutes and then thoroughly shaken for 2 minutes. The volume of each was then recorded and a 3ml sample taken for autoanalysis as described above.

2.3.e. Preparation of DNA standard solutions.

The 0.1 to 1.0μg ml⁻¹ DNA standard solutions were prepared by dilution. A standard solution of 10μg ml⁻¹ Calf thymus DNA in eluting solution was prepared. This was autoclaved for 20 minutes and, once cooled, diluted with eluting solution. Since these studies use a mammalian cell line and given that Hoechst 33258 is sensitive to the G and C base content of DNA⁸ as is the case here a mammalian DNA of very similar G and C content was used for this calibration.
2.3.f. Data Processing.

The integrated peak data for each eluted fraction was corrected for Hoechst 33258 dye–Spectrofluorophotometer response variation with time using the 0.5μg ml⁻¹ response data.

The DNA standard solutions were used to generate a calibration curve of DNA concentration verses fluorescence. Linear regression was then carried out using MINITAB (Minitab Inc) data processing software on the university’s DEC 8650 Vax mainframe computer.

The total amount of DNA in each eluted fraction and the total amount of DNA per filter prior to elution were calculated using a computer program written by Dr B.W. Taylor then of this department. The data was expressed as the proportion of DNA retained on the filter relative to the eluted volume.
References for Chapter 2.


7.


12. Y. Goward, personal communication.

CHAPTER 3. THE EFFECTS OF HYDROXYL RADICAL SCAVENGERS ON RADIATION DAMAGE TO DNA.

3.1. Introduction.

As discussed in Chapter 1, it is commonly thought that damage to DNA as a result of exposure to ionizing radiation leads to cell death. In vitro studies have shown there are two component mechanisms for primary damage to DNA; the direct ionization by interaction of radiation quanta or particles and DNA, and indirect damage caused by attack from water radiolysis products, principally hydroxide radicals (OH), solvated electrons (e^-) and hydrogen atoms (H). It is OH radicals that are generally thought to be of major significance in indirect DNA damage.

The rate constants of DNA attack by OH radicals measured in pulse radiolysis studies of aqueous DNA suggest that *circa* 20% of attack is on the deoxyribose units. This leads to a reasonable mechanism for strand breakage. These, and more recent studies involving model compounds have shown OH radical attack on the base residues is most significant. OH adduct formation at a base residue and the subsequent elimination of H,O followed by hydrogen atom transfer from a neighbouring deoxyribose unit gives a reasonable mechanism ultimately leading to strand breakage. This reaction, which is probably endothermic, is justified by the close location to the reaction centre of C–H hydrogen atoms, and that the resulting highly unstable deoxyribose centred radicals can react further to form stable breakdown products.
Whilst the indirect mechanism is thought to dominate in fluid aqueous DNA systems, the direct mechanism is believed to dominate in frozen aqueous DNA systems. The DNA environment in the cell nucleus is somewhere between these two extremes. The highly organised and tightly packed structure of cellular DNA restricts the volume of solvating water around the DNA. Nuclear water also contains a wide range of biomolecules in relatively high concentration which are highly reactive towards any hydrolysis products. Thus only solvent radicals generated in close proximity to 'exposed' DNA may cause damage. Indirect damage is limited by these two factors and direct damage to DNA more significantly in the cell.

Using the plasmid DNA strand break assay technique, Wren observed no reduction in DNA strand breaks when tertiary butyl alcohol (TBA) was used as an OH\(^+\) radical scavenger at concentrations ranging from 1 to 100mM after gamma-irradiation at 77K. He concluded that within the DNA solvating region in frozen aqueous DNA samples, significant quantities of OH\(^+\) radicals are not produced and hence are not responsible for DNA damage in such systems\(^{15}\).

The aim of the studies reported here was firstly to provide further evidence that OH\(^+\) radicals are not responsible for the observed radiation-induced DNA damage in frozen systems, and secondly to elucidate any influence OH\(^+\) radical scavengers may have upon direct radiation damage to DNA.

3.1.a. Dimethylsulphoxide, DMSO.

DMSO is an aprotic polar solvent. Its unique properties give many important and interesting chemical and biological effects. It is favoured with high diffusion rates
across cell membranes and is of great importance as an additive to cellular systems since it is commonly used as a cryoprotector.

DMSO also functions as a radioprotector. Ashwood-Smith demonstrated that DMSO, increased the surviving fraction of mice exposed to lethal dose X-rays\textsuperscript{16}. In the same study both the oxidation product (dimethylsulfone) and the reduction product (dimethylsulfide) were found to have no appreciable action. This suggested strongly that the radioprotective effects were derived from DMSO and not its obvious metabolic products.

DMSO has also been shown to suppress radiation-induced transformation \textit{in vitro}\textsuperscript{17}. These suppressive effects were believed to be the consequence of both free radical scavenging by DMSO molecules and DMSO induced solvent structure alteration. The latter has also been proposed as a mechanism by which DMSO affords the cryoprotection of biomolecules\textsuperscript{18}.

Chapman and co-workers have demonstrated \textit{in vitro} radioprotective effects of DMSO upon the surviving fraction of Chinese hamster fibroblasts after exposure to X-rays, both in the presence and less effectively in the absence of oxygen\textsuperscript{19-21}. These protective effects were believed to be predominantly due to hydroxyl radical scavenging.

Radford using neutral filter elution, demonstrated radioprotection of frozen Mouse L cells from X-radiation induced DNA double strand breaks by DMSO and hence, since this was related linearly to cell survival, from lethal DNA lesions\textsuperscript{22}.

DMSO is an extremely efficient free radical scavenger, and thus is now commonly used as a probe molecule in studies of free radical reactions in biological systems.

DMSO reacts with OH radicals at close to diffusion controlled rates to form the
sulphuranyl radical adduct (Figure 3–1). The sulphuranyl radical is highly unstable and rapidly decomposes to yield a methyl radical\textsuperscript{[23,24]} (Reaction 1). As a result DMSO is widely used in radiation chemistry specifically to scavenge OH radicals\textsuperscript{[25,26]}.

![Figure 3–1. Structure of the sulphuranyl radical adduct formed by OH radical scavenging by DMSO\textsuperscript{[29].}](image)

\[
\text{(Me)\textsubscript{2}SO + OH } \rightarrow \text{(Me)\textsubscript{2}S(O)OH } \rightarrow \text{MeS(O)OH + Me (1)}
\]

**3.1.b. The radiation chemistry of DMSO.**

The radiation chemistry of DMSO itself has not been extensively studied. Williams and co-workers carried out the most significant solid state studies using dilute (\textit{circa.} 1\%) (H\textsubscript{3}C)\textsubscript{2}SO in (D\textsubscript{3})\textsubscript{2}SO, showing that electron capture leads to the formation of methyl radicals (H\textsubscript{3}C) and the anion MeSO\textsuperscript{[27]}\textsuperscript{-}. The expected radical anion is highly unstable and remained undetected\textsuperscript{[28,29]} (Reaction 2).

\[
\text{(Me)\textsubscript{2}SO + e\textsuperscript{-} } \rightarrow \text{[ (Me)\textsubscript{2}SO ]\textsuperscript{-} } \rightarrow \text{Me\textsuperscript{-} + MeSO\textsuperscript{-} (2)}
\]
No methyl radicals were detected in pure solid DMSO irradiated at 77K, however a
carbon centred triplet feature was detected and assigned to H₂C=S(Me)O radicals.
This is believed to be the product of rapid hydrogen atom abstraction by methyl
radicals from neighbouring molecules (Reaction 3).

\[ \text{Me} + (\text{Me})₂\text{SO} \rightarrow \text{CH}_4 + \text{H}_2\text{C-S(Me)}O \quad (3) \]

Deuterium abstraction at 77K is very slow when methyl radicals are generated in
(D₃C)₂SO and thus methyl radicals remain trapped.

Optical pulse radiolysis studies with aqueous DMSO in contrast, have shown that
solvated electrons are the major product\(^{24}\). This was also found to be the case in the
pure liquid\(^{30-32}\). Reaction of solvated electrons and DMSO is relatively slow ( \(k = 1.7\)
\times 10^6 \text{Imol}^{-1}\text{s}^{-1}\)\(^{24}\) and yields a species of \(\text{\max}\) at 350nm, which is believed to be the
anion \((\text{Me})₂\text{SO}^−\). In the pure liquid this is not the case where a transient species of \(\text{\max}\)
at 600nm was observed, this being either the parent radical cation, \((\text{Me})₂\text{SO}^+\) or some
species derived therefrom\(^{30}\).

The radical cation has since been unambiguously observed by esr spectroscopy in
dilute DMSO in CFCl₃ gamma–irradiated at 77K. The cation is characterised by a
septet feature of large g–tensor shift\(^{33}\), due to spin localisation on the sulphur atom.
3.1.c. t-butyl alcohol, TBA.

T–butyl alcohol (TBA) is also commonly used as an OH radical scavenger in radiation chemistry\textsuperscript{[34,35]}. Whilst reacting efficiently with OH radicals in aqueous solution, reaction with solvated electrons is unimportant. In the pure solid, TBA reacts with ejected electrons to form t–butyl radicals (reaction 4).

\[(\text{CH}_3)_3\text{COH} + e^- \rightarrow (\text{CH}_3)_3\text{C} + \text{OH}^- \quad (4)\]

The radical cation may deprotonate and rearrange to form \(\text{H}_2\text{C}–\text{C}((\text{Me})_3\text{C})_2\text{OH}\) radicals (Reaction 5), or release a methyl radical and acetone (Reaction 6)\textsuperscript{[36]}.\n
\[(\text{Me})_3\text{COH}^+ \rightarrow (\text{Me})_3\text{CO} \rightarrow \text{H}_2\text{C}–\text{C}((\text{Me})_2\text{CO}) \quad (5)\]

\[(\text{Me})_3\text{COH}^+ \rightarrow (\text{Me})_3\text{CO} \rightarrow \text{Me}^+ + (\text{Me})_2\text{CO} \quad (6)\]

Reaction with OH radicals is extremely fast and results in hydrogen atom abstraction from the methyl groups to yield \(\text{H}_2\text{C}–\text{C}((\text{Me})_2\text{OH}\) radicals.
Figure 3-2. The first derivative X-band esr of DMSO irradiated at 77K in CD$_3$OD/D$_2$O glass, containing features assigned to Me and H$_2$C-S(Me)O radicals.

mM DMSO solutions giving DMSO to DNA base pair ratios of 1:20, 1:50 and 1:200 respectively. The samples were degassed with respect to oxygen, frozen, then gamma-irradiated at 77K simultaneously with an aqueous DNA control.

No reduction in DNA primary radical yield relative to that of the control was observed in the esr spectra of any of these samples. At 77K the spectra were almost identical to that of the control. This was also found to be the case after annealing to 130K. The measured yields of TH radicals formed on annealing were also found to be
very similar to that of the control sample. This is illustrated by a plot of total DNA primary radical yield v. temperature (Figure 3–3).

Figure 3–3. A plot of total DNA primary radical yield v. temperature for an aqueous DNA control and a sample of DMSO to DNA base pair ratio of 1:20, demonstrating no significant difference in DNA primary radical yield from the control.

For DMSO to DNA base pair ratios of as high as unity, the observed DNA primary radical yields were found to be almost identical to the aqueous DNA control. However for the 0.076M sample (1:1), a weak doublet feature of 23G splitting centred about
free spin was apparent in the esr spectrum superimposed over the DNA and OH·
radical features. This became more clearly defined after annealing to 130K. The
doublet features were irreversibly lost after annealing to 150K, and were thought to be
the central features of the quartet assigned to methyl radicals above.

DMSO concentrations producing DMSO to DNA base ratios of greater than one
however give rise to weak concentration–dependent radioprotective effects.

A series of 50mg ml⁻¹ DNA samples were prepared and irradiated in DMSO
solutions giving DMSO to DNA base pair ratios of greater than one. When recorded at
77K, the esr spectrum of the 10:1 DMSO to DNA base pairs clearly demonstrated a
significant reduction in the intensity of the DNA primary radical features. This
reduction was quantified after annealing to 130K (to allow the OH radical derived
features to be lost) by computer subtraction of the spectrum obtained from the
equivalent aqueous DMSO (0.758M) control at the same temperature. The reduced
yield was found to be 61% that of the control, the loss of the central inflection from
the product spectrum suggesting that this was largely due to reduced yields of the
pyrimidine anion centres C⁻/T⁻ (Figure 3–4.ii).

This reduction in DNA primary radical yield was accompanied by the presence of a
sharp well defined 1:3:3:1 quartet (Aiso = 23G), which was unambiguously assigned to
methyl radicals and a broad 1:2:1 triplet assigned to H₂C–S(Me)O radicals (Figure
3–4.i). These features became more clearly resolved after the irreversible loss of OH
radicals.

The more reactive methyl radicals are irreversibly lost after annealing to 150K.
However, the triplet features assigned to H₂C–S(Me)O radicals are still present in the
esr spectrum after annealing to 170K, all be it at reduced intensity (Figure 3-5), but are lost after annealing further.

Figure 3-4.i) The first derivative X-band esr spectrum of 10:1 DMSO to DNA base pair sample after annealing to 130K, containing features assigned to Me and \( H_2C-S(Me)O \) radicals.

ii) After computer subtraction of the spectrum of 0.758M DMSO again at 130K to reveal the composite central features assigned to DNA primary radicals, albeit with reduced contribution from the doublet features assigned to the pyrimidine anions.
Figure 3–5. The first derivative X–band esr spectrum of 10:1 DMSO to DNA base pairs after annealing to 170K.

On annealing it is also clear that the reduced yield of the pyrimidine anion centres C⁻/T⁻ is accompanied by a similarly reduced TH radical yield. At 190K, the TH radical yield was found to be 59% that of the aqueous DNA control.

The concentration dependence of this radioprotective effect is demonstrated in both; i) a plot of TH radical yield versus temperature for a range of DMSO to DNA base pair ratios (figure 3–6.i.), and ii)a plot of DNA primary radical yield at 130K versus DMSO concentration (Figure 3–6.ii).
Figure 3-6.i) A plot of TH radical yield versus temperature for a range of DMSO to DNA base pair ratios.
ii) A plot of DNA primary radical yield at 130K versus DMSO concentration.
3.2.c. t-butyl alcohol–DNA systems.

Again based on additive to DNA base pair ratios used in previous studies, aqueous 50 mg ml\(^{-1}\) DNA samples were prepared in 1.52, 3.78, 7.58 and 75.75 mM t-butyl alcohol (TBA) solutions, resulting in TBA to DNA base pair ratios of 1:50, 1:20, 1:10 and 1:1. These samples were then degassed with respect to oxygen and irradiated at 77K.

Similarly to the DMSO systems of comparable concentrations, no detectable difference in the yield of DNA primary radicals was observed in the esr spectra of any of these samples at 77K. On annealing to 130K, the observed spectra were effectively identical to that of the control.

Though TBA derived radicals may have been expected, no such species were detected. Even trace amounts of methyl(CH\(_3\)) and tertiary butyl ((Me)\(_3\)C) radicals, whose narrow features may be detected at very low concentrations, were not detected.

However the effects of higher TBA concentrations are in marked contrast to those observed with the equivalent DMSO concentrations. DNA samples containing 10:1 and 20:1 TBA to DNA base pair ratios demonstrated linear concentration dependent radiosensitizing effects in that DNA primary radical yields are lineally increased with increase in TBA to DNA base pair ratio.

Again no TBA derived features were detected in the esr spectra, despite the use of high gain, narrow field modulation and multiple accumulations to optimise the resolution of any narrow features. This was also found to be the case after annealing to 130 K.
On further annealing it is clear that the increased DNA primary radical yield is also reflected in proportionally increased TH radical yields (Figure 3-7).

Figure 3-7. i) First derivative X-band ESR spectrum for TBA-DNA (20:1) sample after exposure to gamma-radiation at 77K and annealing to 210K, showing features assigned to TH radicals. The intensity of these features is circa twice that of the control.

ii) Effect of TBA concentration (TBA : DNA base pairs) on the intensities of DNA primary radical features at 150K and TH radicals at 170K (--- TH, ——— DNA).
3.3. Discussion.

At low concentrations, giving additive to DNA base pair ratios of less than 1:1 both DMSO and TBA gave no measurable reduction in DNA primary radical or TH radical yields. As stated above, electron- donor and acceptor systems have previously been shown to produce clear DNA radical yield reductions at concentrations giving additive to DNA base pair ratios of 1:50.

These results support the contention that OH radical reactions, in marked contrast to liquid aqueous systems, are not an important source of DNA damage in frozen aqueous conditions.

The esr spectra of samples containing high DMSO concentrations contain features assigned to Me and H$_2$C-S(Me)O radicals which were also observed in the equivalent aqueous DMSO samples. However, since their appearance is accompanied by significant reductions in both DNA primary radical and on annealing TH radical yields, these features are not simply derived from radicals formed in a separate DMSO–H$_2$O phase.

The ionization potential of DMSO is 9.1 eV, which is greater than that of guanine (8.24 eV). Thus hole transfer from G$^*$ to DMSO should not be favoured and thus not contribute to the observed reduction in DNA primary radical yield. DMSO radicals are to be expected at high concentrations, however DMSO was shown to be ineffective at low concentrations.

Several factors probably contribute to the overall reduction in DNA radical yields. The reduction in water molecule concentration in the solvating region surrounding the DNA caused by the presence of DMSO molecules may facilitate charge...
recombination or electron return, especially by the suppression of protonation from the solvent.

The reaction of H$_2$O$^+$ described in chapter 1, to give ultimately G$^+$ may be less favoured than reaction with DMSO to yield DMSO$^+$. DMSO molecules may also be expected to react with electrons thus preventing the formation of the pyrimidine anions T$^−$/C$^−$. They may potentially also scavenge electrons from the pyrimidine anion centres before protonation.

Electron transfer between DMSO molecules may also remove electrons from close vicinity to the DNA and thus reduce the yield of pyrimidine anion centres.

Whilst the effects of low TBA concentrations correlate closely to those observed with similar DMSO concentrations, the effects of high TBA concentrations are in marked contrast with those of high DMSO concentrations. This may be understood in the following way. In both cases, on freezing the co-solvent molecules are forced into close proximity to the DNA, thus increasing the DNA target volume (this effect is clearly defined for inert salt systems$^{37}$). Both electron and hole capture by TBA are clearly inefficient, and insignificant here, so both the hole centres and the electrons formed in this increased target volume may reach the DNA, thus increasing the DNA radical yields.

The rates of both electron and hole capture with DMSO are sufficiently high that competition with the DNA can occur. Thus at high concentrations DMSO may dominate and act as an, albeit weak, radioprotector.


CHAPTER 4 - THE INFLUENCE OF BUFFER IONS ON RADIATION DAMAGE TO DEOXYRIBONUCLEIC ACID

4.1. Introduction.

The studies presented in this chapter are centred on the influence that buffering ions may have on direct damage to DNA by ionizing radiation. At pH values greater than pH 3, primary radical yields for water radiolysis at room temperature are essentially independent of pH\(^1\). But for such reasons as sample fragility, ease of sample preparation and ease of manipulation, aqueous DNA irradiation in several commonly used techniques is often carried out in solutions containing complex mixtures of buffering ions. The DNA concentrations are low relative to the concentration of buffering ions.

As described in Chapter 1, in aqueous DNA solutions the formation of ice crystallites on freezing, results in the formation of a 'glass' region as an extended solvation shell to the DNA. High relative buffer ion concentrations result in a much larger 'glass' region as many more buffer ions are forced into close proximity to the DNA. Since these frozen systems are used to study direct damage to DNA by ionizing radiation, it is important to understand any influence that buffer ions and similar additives may have upon:

i) the mechanisms of direct damage.

ii) the action of radiosensitizing and radioprotecting compounds.

The effects of two commonly used buffers are reported here; TRIS hydrochloride, \((\text{HOH}_2\text{C})_3\text{CNH}_2\text{HCl}\), and the hydrogen phosphate buffer system of potassium
dihydrogen orthophosphate (KH$_2$PO$_4$) and disodium hydrogen orthophosphate, (Na$_2$HPO$_4$) at pH 7.4. Also studied were the effects of sulphate ions in equivalent concentrations. The sulphate radical SO$_4^{-}$, generated by the radiolysis of persulphate ions, has been extensively used to probe the mechanisms of direct DNA damage, in recent studies with model systems$^{[2-15]}$. Also given that the radiation chemistry of sulphate ions is well understood, sulphate ions were used to provide a comparison with phosphate ions.

In plasmid DNA strand break assay experiments, where sample irradiation is carried out at 77K, the buffer ion to DNA base pair ratio is commonly of the order of hundreds. Due to the experimental constraints of sample size and esr spectrometer sensitivity, buffer ion to DNA base pair ratios of this order are not reasonable. A ratio of 20:1 was felt to provide an increase in DNA solvation volume, whilst not reducing experimental sensitivity by reducing the DNA content of each sample.

Glasses are infinitely viscous liquids and hence retain liquid phase structure, for this reason glass systems are commonly used in esr spectroscopy. Buffered systems produce similar solvation conditions in the DNA solvation shell when frozen. For this reason and further comparison, sodium perchlorate a commonly used glass forming agent was also included in these studies.
4.2. Results.

4.2.a. TRIS hydrochloride Studies

Gamma-irradiation at 77K of the pure solid \((\text{HOH}_2\text{C})_3\text{CNH}_2\cdot\text{HCl}\) yielded a first derivative esr spectrum, comprising of an 18G doublet centred about free spin (Figure 4–1.i.).

This doublet was also observed after irradiation of frozen aqueous TRIS.HCl solution. This was further found to be the case when both protonated and amine forms were irradiated in lithium chloride glass, although features for the electron loss centre \(\text{Cl}_2^-\) were also observed (Figure 4–1.ii.).

In an attempt to gain further information, TRIS was also irradiated in DCl/D\(_2\)O at 77K. Whilst yielding features for \(\text{Cl}_2^-\), \text{ClOH}^-\) and \(\text{D}^\cdot\) radicals, the sample only produced a more clearly resolved doublet again of 18G (Figure 4.1.iii.).

These uninformative spectra lead to the conclusion that in any TRIS derived radical product, spin cannot be centred on the tertiary carbon (with NH\(_3\) as a leaving group) (Reaction 1), as might be expected from electron capture. Spin centred on Nitrogen, as might be expected from electron loss, also clearly is not observed.

It therefore seems likely that a rapid rearrangement following both electron loss and capture takes place even at 77K. There are two likely mechanisms of electron capture after irradiation:
\[ R= -\text{CH}_2\text{OH} \]

\[
\begin{align*}
R_3\text{C}-\text{NH}^+_3 + e^- & \longrightarrow R_3\text{C} + \text{NH}_3 \quad (1) \\
(\text{I}) \\
R_3\text{C}-\text{NH}^+_3 + e^- & \longrightarrow R_3\text{CNH}_2 + \text{H} \quad (2)
\end{align*}
\]

It might be expected that the rate constant for reaction 1 would be greater than that for reaction 2. However, in the 1,3,4 triol-tertiary butyl radical (I) the unpaired electron would experience hyperfine coupling to six beta-hydrogen nuclei and yield a complex multiplet in esr spectra. This clearly is not observed and suggests that reaction 2 may occur preferentially as the mechanism of electron capture. This is further supported by the possible subsequent reaction of the product hydrogen atom which may abstract a hydrogen atom from a neighbouring TRIS molecule:

\[
\begin{align*}
\text{H} + (\text{HOCH}_2)_3\text{C}-\text{NH}^+_3 & \longrightarrow (\text{HOCH}_2)_3\text{C}-\text{N}^+\text{H}_2 + \text{H}_2 \quad (3) \\
\text{H} + (\text{HOCH}_2)_3\text{C}-\text{NH}^+_3 & \longrightarrow (\text{HOCH}_2)_2(\text{NH}^+_3)\text{C}-\text{C} \text{HOH} + \text{H}_2 \quad (4)
\end{align*}
\]

(The)

The highly labile hydrogen atom would be expected to favour reaction 4 thermodynamically because of steric effects. The observed 18G doublet is likely to be due to radical (II) where spin density is centred on the carbon atom with hyperfine coupling to a single -hydrogen atom.

Electron loss may proceed by the following mechanism (Reaction 5):

74
(HOCH₂)₂C-NH₃ ----> (HOCH₂)(NH'₃)CCH₂O'H + e⁻  (5)

(III)

(HOCH₂)(NH'₃)CCH₂O'H ----> (HOCH₂)(N'H₃)CCHOH + H⁺  (6)

(II)

the resulting radical (III) from reaction 5 is expected to rearrange whilst trapped in the frozen liquid structure of the glass-like phase to again produce radical (II).

The unpaired electron of radical (II) would be expected to occupy a mainly p-character orbital. However because of the high electronegativity of the oxygen atom, the radical is slightly distorted from planarity to being pyramidal with the introduction of more s-character into this orbital. Coupling to one -hydrogen atom gives rise to the observed doublet A₁₈₀ of 18G.
Figure 4–1. The first derivative X–band esr spectra of gamma–irradiated; i) TRIS hydrochloride solid at 77K, ii) TRIS in 10M LiCl solution at 77K and iii. TRIS in DCl/D$_2$O at 77K, all three spectra contain an 18G doublet feature assigned to (HOCH$_2$)$_2$(NH$_3^+$)CC-HOH radicals.
4.2.b. TRIS–DNA systems.

25 mg ml\(^{-1}\) solutions of DNA were prepared giving final TRIS to DNA base pair ratios of 1:20, 1:10, 1:1 and 10:1. These solutions, once degassed to remove any oxygen, were formed into uniform frozen samples and gamma–irradiated for 4 h at 77K simultaneously with a DNA control to allow quantitative comparison of radical yields.

The samples of 1:20, 1:10 and 1:1 TRIS DNA base pair ratios produced first derivative X–band esr spectra that were almost identical to that of the DNA control at 77K. On annealing this, equality in primary radical yield is further emphasised by similar yields of TH\(^{+}\) radicals.

However, the spectra for samples of 10:1 TRIS to DNA base pair ratios and greater differed significantly from the control. At 77K in both cases the central inflection of the DNA radical anion T\(^{-}\)/C\(^{-}\) was clearly enhanced, suggesting an increased radical anion yield and relative contribution to this feature. The total increase in yield

![Figure 4–2. First derivative X–band esr spectrum of TRIS 10:1 DNA pairs at 77K, demonstrating a substantial enhancement in the doublet component of the DNA primary radical features, hence an enhancement in DNA radical anions.](image-url)
appeared to be only from an increase in anion radical yield. The yield of G\(^-\) appeared to be unaltered; this was supported by comparison with computer simulations (Figure 4–2).

Unfortunately, the magnitude of this enhancement could not be accurately measured because the central feature also contains a contribution from the TRIS derived radical \((\text{HOCH}_2\text{(NH}_3\text{)})(\text{C}_2\text{H}_4)\text{CHOH}\).

However on annealing, it was clear that TH yields in both cases are significantly

Figure 4–3. Plot of TH radical yield for Tris to DNA base pair ratios of 1:10, 1:1 and 10:1 versus temperature, demonstrating great enhancement of TH radical yield in the 10:1 sample.
larger than in the DNA control. Comparison of the spectra recorded after annealing to 190K demonstrates the enhancement in yield to be circa 260%. This is illustrated by a plot of TH yield versus temperature for these samples (Figure 4.3.).

The increase in DNA radical anion yield and consequently in TH yield, suggests that whilst electron capture by TRIS may occur, the rate constant for electron capture by the DNA is greater than that for electron capture by TRIS.

There appeared to be no enhancement in G' yield. Hole transfer to the DNA from the TRIS electron loss product radical (III) in reaction (5) is possible by electron transfer. However the formation of the \((\text{HOCH}_2\text{NH}_3\text{CH}_3\text{OH})\) radical is likely to be very fast and appears to prevent any enhancement in G' yield.

The increase in DNA primary radical anion yield observed at high TRIS concentration may be explained in the following way: The DNA target volume is increased relative to the control by the inclusion of a large number of buffer ions in the DNA solvation shell, the DNA competes favourably for electron capture resulting in the observed enhanced radical anion yield.

4.2.c. Phosphate in \(\text{H}_2\text{O}\).

In order to obtain the buffered pH of 7.4, a ratio of 1:4.1 for potassium dihydrogen orthophosphate \((\text{KH}_2\text{PO}_4)\) and disodium hydrogen orthophosphate \((\text{Na}_2\text{HPO}_4)\) respectively was used to give a final total \(\text{HPO}_4^{2-}\) concentration in water of 0.75M.

The first derivative esr spectra recorded after gamma-irradiation at 77k contains a doublet feature assigned to hydrogen atoms, \(^1\text{H} \cdot \Delta_{\text{iso}}\) for this doublet being 507.5G centred at free spin. The presence of hydrogen atoms in the esr spectrum is a clear
indication that glass regions are present, hydrogen atoms produced in the ice
crystallites react to form hydroxyl radicals even at 77K. Also apparent was a 34G
doublet assigned to the perpendicular features of the well characterised PO₄²⁻
radical[16] superimposed over the expected perpendicular features of OH. It must be
stressed that these features may also be assigned to the protonated radical HPO₄⁻.

Annealing to 130K resulted in the loss of hydroxyl radicals from the ice phase. The
parallel features of the PO₄²⁻ radical become apparent at this temperature, but these

Figure 4–4. First derivative X–band esr spectrum of KH₂PO₄/Na₂HPO₄ (0.75M with
respect to HPO₄⁻ ions and 1:4.1 ratio respectively) solution in H₂O after
gamma–irradiation at 77K and annealing to 130K, showing doublet features assigned
to H (Aₐₒ = 507.5G) and the perpendicular features of PO₄²⁻ radicals.
were poorly defined and the perpendicular features dominated the spectrum. The recorded, \( A_{\text{perp}} = 34 \text{G} \) and \( g_{\text{perp}} \)-tensor = 2.008 agree well with values reported in the literature\(^{[17]}\) (Figure 4-4).

The \( \text{PO}_4^{2-} \) radical has been studied extensively\(^{[18,19]}\). It is a 31-electron species with the unpaired electron confined to non-bonding orbitals on oxygen and has a distorted tetrahedral structure. The degree of distortion is dependent on the degree of delocalisation of the unpaired electron. The electron interacts with the phosphorous nucleus by spin polarisation of the P–O sigma bonds. This coupling is weak results in the small isotropic splitting.

\( g \)-Anisostropy arises as a result of confinement of the unpaired electron to less than all of the ligand oxygen atoms. The largest deviation from free spin would result from confinement to a single oxygen atom. The degree of deviation from free spin is a measure of the degree of confinement.

In the present case, \( g_{\text{perp}} \) was very close to free spin, whilst the parallel features were down field. This apparent axial symmetry suggests considerable localisation on a single oxygen ligand.

The \( \text{PO}_4^{2-} \) radical is thought to be the direct product of electron loss (Reaction 7):

\[
\text{HPO}_4^{2-} \quad \rightarrow \quad (\text{H}^+) + \text{PO}_4^{2-} + e^- \quad (7)
\]

It may also be formed indirectly by electron capture (reaction 8):

\[
\text{HPO}_4^{2-} + e^- \quad \rightarrow \quad \text{P(O}_3\text{OH}) \quad (1)
\]
The resulting phosphoranyl radical (I) has spin centred on the phosphorous atom
and would yield a doublet of approximately 800G. This is clearly not observed
but it may react further to give hydrogen atoms (reaction 9):

\[
\text{HOPO}_3^{2-} \rightarrow \text{PO}_4^{2-} + \text{H} \quad (9)
\]

Hydrogen atoms were observed and may have reacted further to yield the observed
\(\text{PO}_4^{2-}\) radicals:

\[
\text{H} + \text{HPO}_4^{2-} \rightarrow \text{H}_2 + \text{PO}_4^{2-} \quad (10)
\]

The interesting phenomenon of spin-flip satellites was observed in both features of
the 507.5G hydrogen atom doublet (Figure 4–5). Hydrogen atoms trapped in the glass
regions experience the \(1/2\) and \(-1/2\) nuclear spin states of the neighbouring hydrogen
atoms of water molecules. Thus satellite lines were observed at high powers centred
about the esr resonance, with a splitting energetically equivalent to that of the nuclear
magnetic resonance of a proton at this field strength. The narrow lines of the hydrogen
atoms appeared as a doublet of triplets in the esr spectrum (Figure 4–5).

The intensity of these additional features is proportional to a number of factors:
i) the number of neighbouring atoms.
ii) the microwave power of recording.
iii) the inverse of the separation of spin and neighbouring nuclear spin.

At very high powers, further splitting may be observed and the hydrogen atom
resonance appearing as a doublet of quintets.
4.2.d. Phosphate ions in D$_2$O.

The spectrum of the equivalent 0.75 M phosphate D$_2$O sample when recorded at low power (0.01mW) yielded the expected first derivative esr spectra at 77K. The
spectrum contained a 1:1:1 triplet feature assigned to deuterium atoms, \( \text{D} (A_{\text{iso}} = 55\text{G}) \), and features assigned to OD and \( \text{PO}_4^{2-} \) radicals.

The \( \text{PO}_4^{2-} \) features were much better defined at 77K, and after annealing to 130K (Figure 4–6.), in the deuterated solvent. The parallel features were more clearly resolved and the \( g_{\text{para}} \)-tensor was measured. Whilst \( g_{\text{perp}} \) remains very close to free spin, the shift in the \( g_{\text{para}} \)-tensor away from free spin again suggests that the unpaired electron is confined to one oxygen atom, and that the \( \text{PO}_4^{2-} \) radical is distorted.

Figure 4–6. First derivative X-band esr spectrum of \( \text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4 \) (0.75M with respect to \( \text{HPO}_4^{2-} \) ions and 1:4.1 ratio respectively) solution in \( \text{D}_2\text{O} \) after gamma-irradiation at 77K and annealing to 130K, containing well defined perpendicular and parallel doublet features assigned to \( \text{PO}_4^{2-} \) radicals and a triplet feature assigned to D (\( A_{\text{iso}} = 55\text{G} \)). The shift in \( g_{\text{para}} \)-tensor down field of free spin suggesting axial-symmetry and hence spin localisation predominantly on a single oxygen atom. The central feature (D [0]) of the triplet feature assigned to deuterium atoms demonstrating relaxation transfer effects.
Higher power (0.2mW) revealed inequalities in the intensities of the D triplet. This was due to spin relaxation transfer effects. Single resonances may become power saturated at high microwave powers. In this case the D±1 features became partially saturated. Complex resonances however, may exchange some spin state relaxation; the central resonances here, which saturate at relatively high powers, induced spin relaxation in the central D feature and its intensity was thus less diminished by the increased microwave power.

4.2.e. Phosphate DNA Systems.

As described above and in Chapter 2, DNA (25mg ml⁻¹), samples were prepared with an HPO₄²⁻ ion to DNA base pair ratio of 20:1 buffered at pH 7.4 in both H₂O and D₂O solvents. Samples of uniform size were simultaneously gamma-irradiated, positioned accurately in the spectrometer cavity and recorded along with the equivalent phosphate and DNA controls to enable accurate computer subtractions and measurement of DNA radical yields.

At 77K, the uninformative first derivative esr spectrum contained the expected features of hydroxyl (OH) radicals; their yield was diminished in comparison with that of the DNA control. This was probably due to the increased volume of the DNA-solute glass region relative to that of the ice crystallite phase. The −1/2 perpendicular OH feature was also altered in shape when compared with the DNA control due to a contribution from the parallel features of PO₄³⁻ radicals. The central broad feature was a composite of the DNA primary radicals G⁺ and T⁻/C⁻ and the perpendicular features of the PO₄³⁻ radical. Computer subtraction of the spectra
obtained from 0.75M HPO$_4^{2-}$ control (equivalent phosphate concentration) revealed a 60% decrease in DNA primary radical yield when compared with the DNA control.

After annealing to 130K, the poorly resolved parallel features assigned to the PO$_4^{2-}$ radical were apparent (Figure 4–7.i.). For the deuterated solvent computer subtraction of the DNA control confirmed that the central complex resonance was indeed composed of DNA primary radical features and the perpendicular features of the PO$_4^{2-}$ radical (Figure 4–7.ii.).
Figure 4–7.i. First derivative X-band esr spectrum of 20:1 phosphate to DNA base pair ratio sample in H₂O after gamma-irradiation at 77K and annealing to 130K, containing poorly defined parallel features of PO₄²⁻ radicals down field of free spin. ii. The resulting spectrum obtained by subtracting the spectrum of the DNA control from that of the phosphate–DNA sample after annealing to 130K. Clearly defined are both the perpendicular and parallel features assigned to PO₄²⁻ radicals (g_{perp} = 2.014 and g_{para} = 2.032).
There was no measurable increase in the yield of PO$_4^{2-}$ radicals with the loss of OH radicals after annealing. OH radicals might be expected to react with PO$_4^{3-}$ ions to give further PO$_4^{2-}$ radicals and OH$^-$ ions. This supports the premise that all the observed OH radicals are present only in the ice phase and that the vast majority of the solute ions are confined to the DNA–glass and possibly other glass regions.

On further annealing, it was apparent that the reduced yield in DNA primary radicals resulted in reduced yield of TH radicals relative to that of the control. This reduction is best illustrated by a plot of TH yield verses temperature (Figure 4–8).

Figure 4–8. Plot of TH radical yield versus temperature for phosphate–DNA and DNA control samples in H$_2$O, illustrating the reduction in TH radical yield in the phosphate–DNA sample.
At 190K, the poorly defined parallel and well defined 31G splitting perpendicular features of PO$_2^-$ were apparent in the esr spectrum, g$_{\text{exp}}$-tensor being 2.014. Due to poor definition, the g$_{\text{para}}$-tensor could not be accurately measured (Figure 4–9).

All DNA derived features were lost after annealing to 230K, leaving only the features of the PO$_2^-$ radical, from which g$_{\text{para}}$-tensor was estimated to be 2.03. Continued annealing to 270K did not result in the rapid decay of these signals.

After decanting off the liquid nitrogen, the sample was allowed to continue annealing in the spectrometer cavity whilst the spectra was continuously recorded in 30s cycles. This demonstrated the PO$_2^-$ radical only decayed rapidly at the melting point of the system.

Figure 4–9. First derivative X-band esr spectrum of 20:1 phosphate to DNA base pair ratio sample in H$_2$O after gamma-irradiation at 77K and annealing to 190K. Containing the poorly resolved parallel feature and well resolved doublet parallel feature of PO$_2^-$ radicals.
4.2.f. Sulphate ions in H\(_2\)O and D\(_2\)O.

After irradiation at 77K the first derivative esr spectrum for 0.75M sodium sulphate in H\(_2\)O contained features assigned to OH radicals. The existence of a sodium and sulphate ion containing glass region is demonstrated by the presence of a 508G doublet assigned to hydrogen atoms in the esr spectrum. The central feature is also enhanced by an unknown resonance centred very close to free spin. After annealing to 130K, the loss of ice phase derived OH radical features allowed this sharp central feature to be characterised as SO\(_3^-\) radical anions, the recorded g–tensor \(g = 2.0036\), being in good agreement with the published value\(^{18}\).

SO\(_3^-\) radicals are believed to be the product of electron capture by sulphate ions

(Reaction 11):

\[
\text{HSO}_4^- + e^- \rightarrow \text{HSO}_4^{2-} \rightarrow \text{SO}_3^- + \text{OH}^- \quad (11)
\]

Features down field of free spin were assigned to the electron loss product SO\(_4^{2-}\) radical anions (Reaction 12):

\[
\text{SO}_4^{2-} \rightarrow \text{SO}_4^- + e^- \quad (12)
\]

the resulting 'hole' may be centred on one, two or three oxygen atoms and hence gives rise to three g tensors; \(g_x\), \(g_y\), and \(g_z\), the \(g_z\) tensor giving the greatest chemical shift from free spin, the \(g_x\) tensor being very close to free spin and obscured by the intense central feature of SO\(_3^-\) radicals. These features were more clearly resolved in the
spectrum of the deuterated solvent sample (Figure 4–10), where $g_\alpha = 2.034$ and $g_\gamma = 2.02$.

An unassigned feature at $g = 1.996$ was also observed at 130K having been gained on annealing, this was also observed in both solvents and remains uncharacterised.

Figure 4–10. First derivative X-band esr spectrum of 0.75M Na$_2$SO$_4$ solution in D$_2$O after gamma-irradiation at 77K and annealing to 130K, containing features assigned to SO$_3^-$ ($g_\alpha = 2.0036$) and SO$_4^-$ ($g_\alpha = 2.034$, $g_\gamma = 2.02$) radicals.
4.2.g. DNA–Sulphate systems.

25mg ml⁻¹ DNA samples were prepared in 0.75M sodium sulphate solutions in both H₂O and D₂O, these were degassed, frozen and irradiated as described above.

At 77K, the first derivative esr spectrum of the H₂O sample contained the expected features assigned to DNA primary radicals and OH⁻ radicals. In addition a sharp singlet feature was also apparent at g = 2.0036, this was again assigned to SO₃⁻ radicals as above. The same spectrum recorded at 800G contained a 507G doublet assigned to hydrogen atoms, demonstrating the existence of glass-like regions.

![Graph](image)

Figure 4–11. The resulting spectrum obtained by subtracting the spectrum of the DNA control from that of the sulphate–DNA sample (both samples in D₂O) after annealing to 130K. The spectrum contains features assigned to SO₃⁻ and SO₄⁻ radicals.

Annealing to 130K resulted in the loss of OH and H features, and OD and D features from the spectra of the H₂O and D₂O samples respectively. In the spectra of
both samples, the feature assigned to SO$_3^-$ radicals was more clearly resolved than at 77K. Computer subtraction of the spectrum of the DNA control(D$_2$O) from that of the sulphate–DNA(D$_2$O) sample revealed features assigned to SO$_4^-$ radicals (Figure 4–11).

On further annealing of the H$_2$O sample it became clear that whilst TH features were observed, with the corresponding loss of intensity in the DNA primary radical features, the TH yield was significantly reduced in comparison with that of the DNA control. Presumably this was due to the competition for electron capture by sulphate ions, resulting in the observed SO$_3^-$ radicals.

At 210K the yield of TH was found to be significantly less than that of the DNA control, this is illustrated by a plot of TH yield versus temperature (Figure 4–12). The features assigned to SO$_3^-$ and SO$_4^-$ radicals were still detected in spectra recorded at 270K (although the total number of spins was only ca. 30% that of the equivalent phosphate sample at 270K) and were only lost after thawing.
Figure 4-12. Plot of TH radical yield versus temperature for sulphate-DNA and DNA control samples in H$_2$O, illustrating the reduction in TH radical yield in the sulphate-DNA sample.

4.2.h. Perchlorate ions in H$_2$O and D$_2$O.

The radiation chemistry of perchlorate ions in frozen aqueous systems has been extensively studied, since these ions are commonly used as glass forming agents in esr studies of frozen aqueous systems. 0.75M sodium perchlorate solutions were prepared with H$_2$O and D$_2$O solvents and irradiated at 77K as described above. Both samples produced the expected first derivative esr spectra at 77K.
The spectrum of the perchlorate-\(\text{H}_2\text{O}\) sample contained a 507 G doublet assigned to hydrogen atoms (demonstrating the formation of glass-like regions within each sample even at this relatively low concentration), as well as the parallel features of \(\text{OH}\) radicals. Also present down field of free spin was a broad singlet feature assigned to the perpendicular feature of \(\text{O}^-\) radical anions. The \(g_{\text{par}}\) tensor being 2.08 which corresponds well with literature values\(^{19,20}\). The parallel feature of the \(\text{O}^-\) ions \((g_{\text{para}}\text{ free spin})\) was obscured by the large parallel features of the \(\text{OH}\) radicals.

The \(\text{O}^-\) radical features were more clearly resolved in the 77K spectrum of the sample prepared in \(\text{D}_2\text{O}\), along with with features assigned to deuterium atoms and \(\text{OD}\) radicals (Figure 4–13).

\(\text{O}^-\) radicals are believed to be the product of electron capture by perchlorate ions:

\[
\text{ClO}_4^- + e^- \rightarrow \text{ClO}_4^{2-}
\]

The resulting \(\text{ClO}_4^{2-}\) radicals is extremely unstable in aqueous solution and decay rapidly to yield \(\text{ClO}_3^-\) ions and the observed \(\text{O}^-\) radicals\(^{19}\):

\[
\text{ClO}_4^{2-} \rightarrow \text{ClO}_3^- + \text{O}^-
\]
Figure 4–13. First derivative X–band esr spectrum of 0.75M NaClO₄ solution in D₂O after gamma–irradiation at 77K. The spectrum contains features assigned to O^−, D and OD radicals.

4.2.i. DNA–Perchlorate systems.

25 mg ml⁻¹ DNA samples were prepared in 0.75M sodium perchlorate solutions in both H₂O and D₂O, these were degassed and gamma–irradiated at 77K as described above to allow direct comparison with a DNA control.

At 77K, the first derivative esr spectrum of the H₂O sample contained features assigned to ice phase OH radicals, the DNA primary radical features of G⁺ and T⁻/C⁻. The G⁺ yield was found to be slightly enhanced relative to the DNA control. This enhancement was probably due to 'hole' transfer from the perchlorate glass. Hole
centres are not extensively trapped in perchlorate glasses and may be transferred to
the DNA by the following charge transfer processes:

$$\text{ClO}_4^- + \text{ClO}_4^- \rightarrow \text{ClO}_4^- + \text{ClO}_4^-$$

$$\text{ClO}_4^- + G \rightarrow \text{ClO}_4^- + G^+$$

Also present in the spectrum was the low field perpendicular feature assigned to
O\(^-\) radicals, \(g_{\text{perp}} = 2.08\). These again being the product of electron capture by
perchlorate ions as described above. A singlet feature positioned very close to free
spin was also detected, the intensity of which was found to increase on annealing to
130K. This feature was not due to trapped electrons, since the sample did not appear
to be coloured as might be expected as the result of such trapped centres. Also
'Photobleaching' by exposure of the sample to visible light did not result in any
reduction of the feature's intensity. Thus the feature remained unassigned since the
singlet feature gave little information of the nature of the species responsible. The
unknown radical is termed radical \(\gamma\).

Annealing to 130K resulted in the irreversible loss of the ice phase OH radicals
and the loss of features assigned to O\(^-\) radicals from the spectrum. Features assigned
to DNA primary radicals were present, and as stated above the intensity of the central
singlet feature due to the unknown radical was increased. Also apparent was an
intense, well resolved unassigned 40G doublet centred on free spin, and a poorly
resolved 89G doublet also centred about free spin. These features were more clearly
resolved in the spectrum of the sample prepared in D₂O (Figure 4–14), and were termed radicals α and β respectively.

The three unassigned radicals α, β and γ were gained within the spectra, with the corresponding loss of the features assigned to O⁻ radicals. These radicals have g tensor values very close to free spin, and radicals α and β have hyperfine splitting suggestive of coupling to hydrogen nuclei. Such esr parameters suggest that these radicals have spin density centred on C–H units (22G), this strongly suggests that these features may have been due to trapped sugar centred radicals formed as a result of attack on the DNA by O⁻ radicals. O⁻ radicals are expected to react like OH⁻ radicals given their chemical similarity, thus they might be expected to react with DNA by hydrogen atom abstraction to give sugar radicals and hydroxide ions.

Figure 4–14. First derivative X–band esr spectrum of 20:1 perchlorate to DNA base pair ratio sample in D₂O after gamma–irradiation at 77K and annealing to 130K, containing a singlet feature and two doublet features of the unassigned 'sugar' radicals γ, α(A₁₀₀ = 40G) and β(A₁₀₀ = 86G) respectively.
On annealing to 150K the intensity of these radicals remained constant, the fact that their intensity did not increase again suggests that they were the result of DNA attack by O\textsuperscript{-} radicals.

Further annealing to 170K results in the loss the features of \( \beta \) and \( \gamma \) in the spectra in both solvents, whilst the doublet feature of \( \alpha \) was lost after annealing to 190K. After annealing to this temperature, the yield of TH radicals was found to be reduced in comparison with that of the DNA control. This is illustrated by a plot of TH intensity versus temperature (Figure 4–15) and is thought to be the result of competition for electron capture at the pyrimidine bases by perchlorate ions. Electron capture by the perchlorate ions giving rise to the observed O\textsuperscript{-} radicals.

Figure 4–15. Plot of TH radical yield versus temperature for perchlorate–DNA and DNA control samples in H\textsubscript{2}O, illustrating the reduction in TH radical yield in the perchlorate–DNA sample.
4.3. Discussion.

Fan using the plasmid DNA strand break assay technique with irradiation at 77K demonstrated that, for both single and double strand breaks (in order of effectiveness, greatest to least), phosphate, sulphate and perchlorate ions all give significant radiosensitization relative to plasmid DNA irradiated in the absence of buffering ions\textsuperscript{[21]}. However in the esr results reported here, the DNA primary radical yields are significantly reduced by these ions compared with their yield in the DNA control. After annealing, TH\textsuperscript{-} yields were also found to be similarly reduced. A plot of TH\textsuperscript{-} yield versus temperature (Figure 4–16.i) demonstrates this apparent radioprotective effect.

This paradox may be resolved by considering the fate of the observed inorganic radicals produced by radiolysis in the DNA solvating phase. As described in Chapter 2 relative radical yields throughout the annealing process may be compared by double integration of the recorded first derivative esr spectra (Figure 4–16.ii).

After annealing to 130K, all ice crystallite phase radiolysis products decay and the remaining radical concentrations were due to the DNA and its associated solvation shell. At this temperature there was a significant increase of the order of 160% in the number of radicals present in this solvating glass like phase in the phosphate, sulphate and perchlorate samples compared with that of the DNA control.
Figure 4-16.1. Plot of TH radical yield versus temperature for phosphate–DNA, sulphate–DNA and perchlorate–DNA and DNA control samples in H₂O.

ii. Total radical yield versus temperature for phosphate–DNA, sulphate–DNA and perchlorate–DNA and DNA control samples in H₂O.
As discussed above in chapter 1, for frozen aqueous DNA gamma-irradiation results in damage to the DNA by direct absorption of quanta by the DNA itself and resulting in G^+ and T^-/C^- formation and also in DNA damage by subsequent transfer of radiolysis damage from the solvating region. Thus the solvating region may be described as the overall target volume of the DNA. The inclusion of extra solute ions increases this target volume. The resulting increased number of radiolysis products may react with the DNA providing they are sufficiently mobile to reach the DNA without reacting with other radiolysis products.

Solute radiolysis products formed in the solvating volume may either react with each other by, for example dimerisation, or react with the DNA at either the sugar residues or the bases. The results reported above demonstrate that the degree of transfer of solvation shell damage is highly dependent on the nature of those species formed by irradiation in this solvating region.

In the case of the DNA control, the target volume is at a minimum. DNA damage results from direct absorption of radiation and hole and electron transfer from the solvation shell.

In the perchlorate case however, the target volume is significantly larger due to the inclusion of sodium and perchlorate ions and their associated solvating water molecules. Thus after irradiation the number of radicals potentially capable of attacking the DNA is increased, illustrated by the increased radical concentration at 130K (Figure 4–16.ii) in the perchlorate sample. As described above, electron capture after irradiation produces O^- radicals, which may eventually dimerise or, because of their chemical similarity with OH radicals, may be sufficiently mobile to react with the DNA. O^- radicals would be expected to attack the sugar residues by hydrogen
atom abstraction to yield the observed sugar radicals $\alpha$, $\beta$ and $\gamma$. Thus the increase in DNA target volume results in increased damage to the DNA which is reflected in the plasmid strand break assay results.

It seems likely that similar processes occur in the sulphate and phosphate cases. The reactions of $\text{SO}_4^-$ radicals with DNA model systems have been extensively studied $^{[12]}$, and it is probable that $\text{PO}_4^{2-}$ radicals behave in a similar way.

However, both sulphate and phosphate are more heavily solvated than perchlorate ions in water, with solvation numbers of $\text{circa}8$ and 12 (plus secondary solvation) respectively. Their electron loss and gain products described above are likely to be similarly solvated. This would be expected to substantially reduce their mobility within the glass like solvating region and hence their reactivity at these temperatures, the phosphate derived radicals being the least mobile. As described above, the radiolysis products of both sulphate and phosphate ions were still present in the esr spectra at 270K, the DNA derived radicals decaying below 230K. Any DNA radicals produced at by attack by the observed sulphate or phosphate derived radicals decay too quickly to be trapped and observed by esr spectroscopy at these temperatures.

In the radical concentration versus temperature plot (Figure 4–16.ii), the less heavily solvated and hence more mobile sulphate derived radicals decay at slightly lower temperatures than the $\text{PO}_4^{2-}$ radical, supporting the argument that the degree of solvation at least partially governs reactivity in these systems.

The sulphate radicals may also dimerise to form persulphate:

$$2 \text{SO}_4^- \longrightarrow \left[\text{O}_3\text{S}^-\text{O}^--\text{OSO}_3^-\right]^2^-$$
However $\text{PO}_4^{2-}$ radicals dimerise less readily and are more likely to attack the DNA, ultimately leading to the observed yield strand breaks.

In conclusion, the major increase in DNA strand breaks induced by phosphate buffers must be largely due to the major increase in target volume induced by these glass-forming ions. The contrast between the esr results and the plasmid strand break assay results is largely explicable in terms of the lack of mobility of the phosphate radicals, ascribed to their high degree of solvation, such that esr spectroscopy is unable to detect their ultimate reactions with DNA because the resulting DNA radicals are not trapped at these temperatures.
References for Chapter 4.


CHAPTER 5 - The effects of polyammonium cationic derivatives of 2-nitroimidazole compounds on direct damage to DNA.

5.1. Introduction.

The potent radiosensitizing effects of molecular oxygen are well documented, in that the effects of X-irradiation in the absence of molecular oxygen are much reduced. In fact oxygen is the most powerful radiosensitizer yet discovered. However this is an undesirable phenomenon in the context of cancer radiotherapy, where a tumour must be considered as part of the body and not as an isolated system. Whilst with sufficiently high doses all tumour cells can be killed, the most important consideration is to minimise damage to the surrounding normal tissue, the 'tumour bed', and so ensure its future function.

Reduced blood supply in tumours and high metabolic rates within tumour cells themselves, results in layers of cells within the tumour that are hypoxic. These cells are thus less sensitive to radiation treatment than the surrounding oxic tissues.

Compounds which specifically sensitise hypoxic cells to radiation, whilst not being metabolised themselves, have potentially great value in the clinical treatment of cancer by radiotherapy. Such compounds might allow more efficient tumour cell killing with lower doses of radiation, thus reducing radiation damage to the surrounding normal tissue. For this reason, a great volume of work has been devoted to the phenomenon of radiosensitization and to the development of effective radiosensitizers both in vitro and in vivo.
Radiosensitization of the cell may be brought about in a number of ways; the mimicry of the effects of molecular oxygen, the inhibition of repair processes and the depletion of natural intracellular radioprotectors. For example the drugs \( N\)-acetyl-maleimide (NEM) and diamide inhibit and deplete the non-protein sulphydryl compounds such as glutathione within the cell. Thus both compounds demonstrate radiosensitizing effects in both oxic and hypoxic conditions\(^1\).

A great deal of radiosensitization research has been devoted to the development of drugs which give sensitization by mimicking the effects of oxygen. Initially studies were confined to bacterial systems\(^{2,3}\). The electron affinic compound \( p\)-nitroacetophenone was found to sensitize mammalian cell cultures to the effects of radiation in hypoxic conditions\(^{4,5}\). These findings led to interest in other electron affinic nitro-substituted aromatic compounds particularly heterocycles, for example nitro substituted furans, as potential clinical radiosensitizers\(^6\). However, whilst proving to be effective radiosensitizers in vitro, these compounds were found to be cytotoxic and therefore were unsuitable for clinical applications.

The 5-nitroimidazole, metronidazole (Figure 5-1.i.) was similarly found to sensitize hypoxic mammalian cells to the effects of radiation in vitro\(^7\). Metronidazole had previously been in use clinically for several years as an anti-microbial agent and had proved effective in the treatment of anaerobic microbial infections in wounds and burns, therefore demonstrating a relatively low cytotoxicity. This report led to studies based on a wider range of electron affinic nitroimidazole compounds, leading to the development of more efficient radiosensitizing compounds such as the 2-nitroimidazole, misonidazole (Figure 5-1.ii.)
Metronidazole is believed to exert its anti-microbial effect via reduction of the nitro- group, this is believed to form a reactive intermediate which is thought to disrupt DNA transcription and replication[8]. The ability of nitroimidazoles to sensitize mammalian cells to the effects of radiation in hypoxic conditions has been related to their reduction potential (and hence electron affinity)[9]. In studies using radical scavengers such as cysteamine, the radiosensitizing mechanism of misonidazole has been shown to be a fast process occurring via free radical reactions, and leading to DNA strand breakage[10]. These studies demonstrated that reduced misonidazole yielded DNA strand breaks in alkaline sucrose gradients, although the specific nature of the reduction product was not determined. The radical anions of nitroimidazoles are believed to be a key reduction product produced during radiolysis, these have been extensively studied and characterised by esr and pulse radiolysis techniques[11-13].

A number of mechanisms have been proposed for the radiosensitizing action of nitroimidazoles. Adams[5,14] proposed that following direct ionization of DNA a high degree of charge recombination occurs, and that electron capture by electron affinic drugs, such as nitroimidazoles, may prevent this recombination. This would result in excess DNA electron loss centres and would he proposed, favour the decay of the positive centre to form a neutral free radicals which might decay further to give strand breakage.

Studies have indeed demonstrated that electron capture by metronidazole and misonidazole competes with the formation of DNA radical anions, py−. The metronidazole and misonidazole electron capture products were both found to have spin centred on the nitrogen atom of the nitro- group[13,15]. However in this and previous studies[16], little evidence of charge recombination was found, and plasmid
strand break assays under conditions of direct damage demonstrated radioprotection
by both metronidazole and misonidazole, suggesting the reduction in strand breakage
was the result of reduction in total DNA radical yield due to nitroimidazole
competition for electron capture.

The resulting radical anions MET\(^-\) and MISO\(^-\) were found to relatively stable, it
has been suggested that these may attack the DNA to give sugar centred radicals
which may yield strand breaks\(^{[17]}\). It has also been suggested that further reduction of
these radical anions takes place and that the product nitroxide radicals or
hydroxylamines may be responsible for DNA damage. However, the mechanism by
which electron affinic compounds radiosensitize hypoxic cells still remains
unresolved.

\[
\begin{align*}
\text{Metronidazole} & \quad \text{Misonidazole} \\
\end{align*}
\]

Figure 5-1.i. Metronidazole  ii. Misonidazole
The aim of the studies presented in this chapter, was to elucidate the effect of condensing 2-nitroimidazole compounds into close proximity to the DNA upon radiation induced DNA damage under conditions of direct damage.

As noted above, the site of action within the cell of nitroimidazole radiosensitizers, is believed to be the DNA. Thus the ability to target such radiosensitizers to their site of potential action, offers great advantages in clinical applications.

Polyamines are ubiquitous in living cells and have been implicated in a variety of regulatory processes within the cell\(^{[18,19]}\). At physiological pH, polyamines are fully protonated polycationic species\(^{[20]}\). Polyamines have been shown to bind to the negatively charged phosphate backbone of DNA because of electrostatic forces. This however is not site specific, in fact \(^{23}\)Na and \(^{1}H\) nmr studies of DNA interactions with spermine and spermidine, have shown that such binding is dynamic and that the bound polyammonium cation may migrate laterally along the DNA at close to diffusion controlled rates\(^{[21]}\).

The effects of polyammonium cationic derivatives of two 2-nitroimidazole compounds upon radiation damage to DNA under conditions of direct damage were studied using esr spectroscopy and are reported here. The two 2-nitroimidazole compounds, AM–1229 and AM–1254 (Figures 5–2.i. and ii.), have both previously been shown to bind to DNA\(^{[21]}\), thus the electron affinic nitroimidazole is condensed into close interaction with the DNA. The polyamine substituents were believed not to reduce the electron affinity of these compounds significantly, this has not been quantified however, since their one electron redox potentials have not been measured.
Figure 5-2. i. AM-1229, ii. AM-1254 and iii. spermine.
5.2. Results.

For the initial studies, 50 mg ml\(^{-1}\) DNA samples were prepared with AM 1229 and AM 1254 concentrations of 1.9mM and 3.8mM respectively, giving a final nitroimidazole group to DNA base pair ratio of 1:20 for each sample. The compounds AM 1229 and AM 1254 were prepared and purified by Dr. Andy Mather of this department. For direct comparison, a sample containing the commonly studied 2-nitroimidazole, misonidazole (3.8mM) was also prepared along with a DNA control. These samples were then thoroughly degassed with respect to oxygen and irradiated at 77K as described in Chapter 2.

When recorded at 77K the first derivative esr spectra of all three samples containing nitroimidazole compounds were very similar. The spectra comprised of the expected features assigned to the DNA primary radicals G\(^{+}\) and C\(^{-}/T\(^{-}\) together with features assigned to OH radicals, confined within the phase separated ice crystallites. Also apparent up field of the central DNA features in each sample was the high field M\(_{1}\) = -1 (\(^{14}\)N) parallel feature of a triplet assigned in all three samples to nitroimidazole radical anions (Figure 5-3.i). These radical anions are believed to be the product of electron capture by the nitroimidazole groups, electron density being largely centred on the nitrogen atom of the nitro-substituent, the resulting radical anions being stabilised by delocalisation on the imidazole ring.

After annealing to 130K the OH radicals trapped in the ice crystallites are lost irreversibly. The spectra of the nitroimidazole compound containing samples were again very similar. The esr spectra of all three nitroimidazole samples contain the features of DNA primary radicals (Figure 5-3.ii), however these are significantly
reduced in comparison to those observed in the DNA control. Computer subtraction of
the spectrum of misonidazole prepared in D$_2$O / CD$_3$OD glass enabled the reduction in
DNA primary radical yield to be quantified as being ca. 40%. This appeared to be
predominantly due to the reduction in DNA radical anion centre C$^-$/T$^-$ yields, as a
consequence of the high degree of electron capture by the nitroimidazole compounds,
the G$^+$ yields appeared to remain unaltered. Double integration of the spectra of the
sample containing nitroimidazole compounds and the spectrum of the DNA control
enabled the comparison of total radical yield in each of the samples. These were found
to equivalent within the errors of such integration. Demonstrating that electron capture
by the nitroimidazole compounds competes efficiently with electron capture by the
pyrimidine base residues of the DNA, resulting in reduced DNA radical anion centre
formation. This is in good agreement with previous reported studies$^{[13]}$.

This is further supported by the appearance of clearly defined triplet features
assigned to the nitroimidazole radical anions, where spin is centred largely on the
N-atom of the nitro-substituent of the nitroimidazole group, which are observed in
the esr spectra (Figure 5-3ii).

Further annealing reveals that the reduction in DNA radical anion yield in the
samples containing nitroimidazole compounds is reflected in significantly reduced TH
radical yields. This reduction being ca. 95% in comparison to the DNA control at
210K. The initial reduction in DNA radical anion yield does not account completely
for this large reduction in TH yield suggesting that DNA radical anions C$^-$/T$^-$ are
scavenged by the electron affinic nitroimidazoles before TH formation takes place.
Figure 5-3. First derivative X-band esr spectrum of AM-1229 1:20 DNA pairs at 77K (i), and after annealing to 130K (ii). Both contain features assigned to DNA primary radicals and to the electron capture product, imz–NO$_2^-$ radical anions.
\[ T^- + \text{imz-NO}_2 \rightarrow T + \text{imz-NO}_2^- \]

TH radical yield in these samples is illustrated by plots of the radical yields versus temperature (Figure 5-4).

![Graph showing radical yield versus temperature for different samples.](image)

Figure 5–4. Plot of TH radical yield versus temperature for 1.9mM AM–1229, 3.8mM AM–1254, 3.8mM misonidazole and DNA control samples, illustrating the reduction in TH radical yield in the nitroimidazole containing samples.

Clearly at these concentrations electron capture by the nitroimidazoles occurs so efficiently that little can be gauged from these results concerning the influence of electrostatically binding the nitroimidazole compounds to the DNA.

Thus a further series of 50 mg ml\(^{-1}\) samples were prepared with AM 1254 and misonidazole concentrations of 1.52 mM, and AM 1229 concentration of 0.76 mM,
giving nitroimidazole group to DNA base pair ratios of 1:50. Also prepared, for further comparison, together with a DNA control, was a sample containing a spermine concentration of 0.152 mM. Spermine is a naturally occurring polyamine, and similarly to AM 1229 and AM 1254 possesses three amine groups (Figure 5-2.iii.). These samples were thoroughly degassed with respect to oxygen, frozen and irradiated as described in Chapter 2.

At 77K the spectra of the samples containing AM 1229, AM 1254 and spermine all appeared to be identical to that of the DNA control, consisting of only features assigned to the DNA primary radicals and OH radicals. Only the spectrum of the misonidazole sample contained the high field \( M_0 = -1 \) feature assigned to the nitro-group centred electron capture product, imz\( -\text{NO}_2^- \). After annealing to 130K it became clear that electron capture by the compounds AM 1229 and AM 1254 was significantly less than by misonidazole. Computer subtraction of the spectrum of the DNA control at this temperature from the spectra of the AM 1229 and AM 1254 samples revealed significantly less intense triplet features of the respective imz\( -\text{NO}_2^- \) radicals (Figure 5-5.). The yields of these electron capture products were found to be ca. 8% that of the misonidazole electron capture product.

The yield of DNA primary radicals, in the spermine sample was equal to that of the DNA control, the yield in the AM 1229 and AM 1254 samples were also found to be very similar to that of the control. The misonidazole sample however demonstrated a reduction in DNA primary radical yield of ca. 20% in comparison with that of the DNA control. This appeared to almost wholly consist of reduction in the yield of anion centres, presumably the result of competition for electron capture. The relative yields
of DNA primary radicals versus temperature for these samples are represented in Figure 5-6.

Figure 5-5. The resulting spectrum obtained by subtracting the spectrum of the DNA control from that of the AM-1254 1:50 DNA pairs sample after annealing to 130K. The spectrum contains weak features assigned to imz-NO$_2^-$ radicals.

On further annealing, the spectrum of the misonidazole sample contained prominent triplet feature assigned above to the electron capture product. However only
trace amounts of TH radicals were detected, even at 210K. This suggests that misonidazole not only reduces the yield of DNA primary radical anions, but also scavenges the electron capture centres of the DNA on annealing, even at this relatively low concentration, thus further reducing the TH radical yield.

![Graph](image)

Figure 5–6. Relative yields of DNA primary radicals versus temperature for 0.76 mM AM-1229, 1.52 mM AM-1254, 1.52 mM misonidazole, 1.52 mM spermine and DNA control samples.

Startlingly, considering the very small reduction in DNA primary radical anion yield, a large reduction in TH yield is also observed in the spectra of the AM 1229 and AM 1254 samples in comparison with the yield of the control. In both cases the triplet features of the imz-NO$_2^-$ electron capture product are lost from the spectra after annealing to 150K. This reduction in TH yield was quantified at 210K and was
found to be 80 and 84% for AM 1254 and AM 1229 respectively compared with that of the control.

A reduction in TH was also observed for the spermine sample however this was not so great, giving a 68% reduction at 210K.

These effects are best illustrated by a plot of TH yield versus temperature for the four samples and the control (Figure 5–7).

![Figure 5–7. Plot of TH radical yield versus temperature for 0.76 mM AM–1229, 1.52 mM AM–1254, 1.52 mM misonidazole, 1.52 mM spermine and DNA control samples, illustrating varying reduction in TH radical yields in comparison with the control.](image-url)
5.3. Discussion.

The large reduction in TH yield in the misonidazole case is easily resolvable in the light of the high degree of electron capture. However, the large reduction in TH in the AM 1254 and AM 1229 is less easily explained, since only relatively small yields of their relative electron capture products were detected.

Spermine was also found to reduce TH yield, though less effectively than the nitroimidazole polyammonium cations, AM 1229 and AM 1254. This suggests that in some way the polyammonium cationic species prevent TH formation, possibly by scavenging the DNA radical anion centres. However, polyamines have been shown to be poor electron scavengers, and have been shown to react slowly even with OH radicals\[^{[23]}\]. Thus radical anion scavenging by spermine does seem unlikely and the mechanism by which this suppression in TH yield occurs is unresolved.

It is likely that this unknown mechanism of protection at least partially responsible for the reduction in TH yields observed in the AM 1229 and AM 1254 samples. However there was a greater reduction in yield in these cases particularly. This suggests that the nitroimidazole components also give radioprotective effects. A possible mechanism for this is, that the nitroimidazole components of these compounds may scavenge the DNA radical anions, given their close proximity to the DNA this seems possible. The resulting anion may be sufficiently mobile to progress along the DNA (whilst remaining bound) and return the electron to a 'hole' centre. The anion not being trapped and therefore not observed.
References for Chapter 5.


17. D. Evans, unknown source.


6.1. Introduction.

Neutral (pH 9.6) filter elution is now used extensively to monitor radiation induced DNA double strand breaks both in whole cells and in isolated nuclei. This technique, originally developed by Bradley and Kohn\textsuperscript{11}, allows the quantification of radiation induced damage to DNA at biologically relevant doses and thus allows direct comparison with cell survival studies. Whilst not being devoid of criticism a growing volume of evidence exists establishing the accuracy and precision of the technique\textsuperscript{6-8}.

The aim of the studies presented in this chapter, were to elucidate the effect of condensing the 2-nitroimidazole compound, AM1229 (Figure 5–2) into close proximity to cellular DNA prior to radiation induced DNA double strand breaks as measured by neutral filter elution under conditions of hypoxia.

As noted above, the site of action within the cell of nitroimidazole radiosensitizers, is believed to be the DNA. Thus the ability to target such radiosensitizers to their site of potential action might offer great advantages in clinical applications.

Neutral (pH 9.6) filter elution was used in the studies presented here to measure the relative yield of gamma–radiation induced DNA double strand breaks in Chinese Hamster V79 cells under conditions of hypoxia in the presence and absence of nitroimidazole radiation response modifying compounds. The aim of these studies
was to determine the influence polyamine substituents may have upon the radiation
modifying effects of 2-nitroimidazoles.

It must be stressed that the system used in these studies (described in Chapter 2)
only demonstrated relative radiation effects, since it was not calibrated to give
absolute double strand break yields. For reference, such calibrations may be
performed by using $^{125}$I-dUrd-labelled cells, based on the observation that each $^{125}$I
decay yields on average one DNA double strand break$^{[2]}$. 
6.2. Results.

As described in chapter 2, two identical fractions of Chinese Hamster V79 cell suspensions were prepared, one fraction was retained as a control, the second fraction being dosed with the required compound. These fractions were incubated for 40 minutes while cooling on ice, and then placed again on ice in a continuously purged nitrogen/CO₂ atmosphere for a further 90 minutes to generate hypoxic conditions. After irradiation filter elution was carried out and the results expressed as elution profiles in which the fraction of retained DNA was plotted against the eluted volume.

In order to allow the direct comparison of these elution profiles, the relative strand scission factors for each plot were calculated. The relative strand scission factor being defined as:

\[
\text{relative strand scission} = -\log_{10} \left( \frac{F_x}{F_o} \right)
\]

where; \( F_x \) = the fraction of DNA retained after 21ml of eluted volume of the irradiated sample.

\( F_o \) = the fraction of DNA retained after 21ml of eluted volume of the relative unirradiated control.

Previously the 2-nitroimidazole compound misonidazole has been shown to enhance radiation induced DNA double strand breaks in mouse cells under conditions of hypoxia at concentrations of 10mM\(^{[2]}\). In the same study this dose modifying effect was shown to correlate linearly with radiation induced cell killing. Metronidazole (a
5-nitroimidazole, Figure 5–1), has also been shown to give similar radiosensitizing
effects in terms of radiation induced cell killing under hypoxic conditions\textsuperscript{[3]}. For this
reason an initial experiment was carried out, the test fraction of cells being dosed with
10mM metronidazole. The metronidazole dosed and control aliquots were irradiated at
0, 20, 40 and 80 Gy.

The elution profiles for the control and test fractions are shown in Figure 6–1. To
allow direct comparison, these results are also expressed in a relative strand scission
factor versus dose plot (Figure 6–4). The yield of DNA double strand breaks in the
10mM metronidazole treated cells is enhanced relative to the control cells, the relative
sensitization in strand scission factor being 1.86. Radiosensitizing dose modifying
effects by metronidazole have been shown only to occur in oxygen deficient
conditions, thus this marked sensitization effect demonstrates that hypoxic conditions
were achieved. These results also demonstrate that the system used here is sufficiently
sensitive to monitor such effects.

However, when the experiment was repeated at doses of 0, 5, 10 and 15 Gy, the
scatter in the data was too great to be meaningful. Thus it was felt that the system was
only sufficiently sensitive to measure double strand breaks accurately at doses of 20
Gy and above.

Control fractions and test fractions treated with 5mM AM1229 were irradiated at
doses of 0, 20, 40, 60 and 80 Gy under identical conditions. 5mM AM1229
concentration giving an equivalent nitroimidazole group concentration to that used in
the metronidazole reported above. The elution profile for the AM1229 test cells is
given in Figure 6–2. A plot of relative strand scission factors is given in Figure 6–4,
each point being the mean of three experiments. The measured strand scission factor
at 60 Gy was 0.43 that of the control, demonstrating a marked radioprotective effect under these conditions.

In order to gauge any influence the polyamine substituent of AM1229 might have control fractions and test fractions treated with 5mM spermine were irradiated at doses of 0, 20, 40, 60 and 80 Gy under identical conditions. Spermine having a similar structure to the polyamine substituent of AM1229. The elution profile of the spermine treated fractions is given in Figure 6–3, and the relative strand scission factors (the mean of three experiments) are expressed in Figure 6–4. The measured strand scission factor at 60 Gy was 0.66 that of the control.
Figure 6-1. pH 9.6 neutral filter elution profile of Chinese hamster V79 cells gamma-irradiated under hypoxic conditions. i) control fraction. ii) 10mM metronidazole.
Figure 6-2. pH 9.6 neutral filter elution profile of Chinese hamster V79 cells gamma-irradiated under hypoxic conditions dosed with 5mM AM-1229.

Figure 6-3. pH 9.6 neutral filter elution profile of Chinese hamster V79 cells gamma-irradiated under hypoxic conditions dosed with 5mM Spermine.
Figure 6-4. A plot of relative strand scission factor versus dose.
6.3. Discussion.

Clearly at these concentrations, AM1229 does not give radiosensitizing dose modifying effects in terms of radiation induced DNA double strand breaks under hypoxic conditions, as metronidazole does in these studies and misonidazole have been shown to in previous studies. Instead AM1229 and also spermine give radioprotective dose modifying effects relative to untreated controls under these conditions. The strand scission factor (SSF) at 60 Gy and the sensitization relative to the controls are presented in Table 6–1.

Table 6–1.

<table>
<thead>
<tr>
<th>SSF at 60 Gy</th>
<th>Rel. Sensitization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.181</td>
</tr>
<tr>
<td>Metronidazole (10mM)</td>
<td>0.337</td>
</tr>
<tr>
<td>AM1229 (5mM)</td>
<td>0.078</td>
</tr>
<tr>
<td>Spermine (5mM)</td>
<td>0.120</td>
</tr>
</tbody>
</table>

Thus the effect of electrostatically binding a nitroimidazole compound to DNA in terms of DNA double strand breaks appears to overcome the radiosensitizing action previously observed in studies with these compounds.

Spermine, a polycationic species at physiological pH, also induced radioprotective dose modifying effects. Although to a lesser degree.

These radioprotective effects may be due to hydroxyl radical scavenging by both spermine and AM1229, since a substantial concentration of these DNA binding
compounds might be expected in close proximity to the DNA. AM1229 being a much more efficient OH radical scavenger by virtue of the two nitroimidazole substituents. In previous studies polyamines have been shown to scavenge hydroxyl radicals\textsuperscript{[7]}. However the rate constants for such scavenging are relatively very low, and in biological systems polyamines have been shown to be poor hydroxyl radical scavengers\textsuperscript{[8-10]}. In the previous chapter under conditions of direct damage to DNA, both spermine and AM1229 were shown to significantly reduce the formation of TH radicals. As discussed in Chapter 1, TH radicals are believed to be possible precursors of DNA strand breaks. AM 1229 was observed to give the greater effect. This suggests that, whilst hydroxyl radical scavenging by these compounds may occur, this may not solely responsible for the observed radioprotective effects. In the proceeding chapter it was also suggested that because of electrostatic binding to DNA, that the nitroimidazole component of AM1229 might scavenge trapped anion centres on the DNA formed as a result of direct ionization, and be sufficiently mobile to facilitate electron return to hole centres. Thus DNA bound electron affinic compounds may give rise to repair by facilitating charge recombination, providing they are sufficiently mobile. In room temperature $^{23}$Na and $^1$H nmr studies polyammonium cations have been shown to bind to DNA whilst remaining mobile\textsuperscript{[11]}. The effect of binding a compound known to give radiosensitizing effects, to its expected site of action should simply to enhance such radiosensitizing effects or to at least result in similar radiosensitization. This clearly does not occur here. There is evidence that under hypoxic conditions that some reductive chemical modification of nitroimidazoles takes place, the products of which have been shown to result in
radiosensitizing dose modification effects\cite{12}. Electrostatically binding nitroimidazoles to DNA may remove the imidazole compound from the metabolic processes that result in such reduction, thus preventing radiosensitization. Whilst being a possible reason for the reduction or loss of ability to radiosensitize in terms of DNA double strand breaks, this does not, explain the observed radioprotective effects of AM1229.

In criticism of these experiments additional information may be provided about the mechanism of radioprotection by both AM1229 and spermine by repeating them in oxic conditions. This would also allow an oxygen enhancement ratio measurement of the effects of metronidazole allowing further comparison with other studies and a more accurate assessment of the hypoxic conditions generated here.
References to Chapter 6.


11. K.D. Held and S. Awad., *personal communication*.


Exposure of dilute aqueous DNA to ionizing radiation at ambient temperatures results in indirect damage to the DNA, major reactions being the addition of 'OH radicals to DNA bases and abstraction of C—H hydrogen atoms from deoxyribose units. In order to concentrate on direct damage processes, we have studied frozen aqueous solutions, but it remains possible that some damage is still caused by 'OH radicals generated close to DNA molecules.

In order to check this, we have used two 'OH radical scavengers, dimethyl sulphoxide (DMSO) and t-butyl alcohol (TBA). For both, in low concentrations (< 1 : 1 , base-pair), the damage is confined to DNA and exactly corresponds to normal damage in the absence of scavengers. In a second series of experiments, solid solutions containing hydrogen peroxide were photolysed with UV light at 77 K. This gave a poorly defined sextet assigned to sugar radicals or possibly to thymine 'OH-radical adducts, which was not detected in the absence of hydrogen peroxide, or during γ-radiolysis. We conclude that hydroxyl radical attack is not important for fully hydrated frozen DNA.

The nature and significance of a range of other radicals detected in DNA solutions more concentrated in DMSO and TBA are also discussed, together with the identification of a species giving a broad singlet in the hydrogen peroxide studies.

It is commonly thought that cell death on exposure to ionizing radiation arises primarily as a result of DNA damage. It is therefore important to try to understand the mechanism of radiation damage to DNA. There are clearly two limiting overall mechanisms for primary damage, one being direct interaction between radiation quanta and DNA, the other being indirect damage via attack by water radicals generated by the radiation. In the latter case, damage by hydroxyl ('OH) radicals is thought to be of major significance. Because most studies have been with dilute aqueous solutions in the liquid state, the latter has been far more widely studied than the former. It was originally supposed that 'OH attack was predominantly on the deoxyribose units, especially since this leads to a nice explanation for strand-breaks (SB), but it has been shown recently that, at least with model compounds, addition of 'OH to bases constitutes the major part of 'OH radical attack.1-3 It is suggested that this may be followed by hydrogen-atom transfer from a neighbouring sugar unit, as we postulated originally as part of the direct damage mechanism* (fig. 1). We stress that our justification for such reactions, which are probably endothermic, was that the C—H hydrogen atoms are 'poised' close to the reaction centre, and that after transfer [reaction (1)] the reactive sugar radicals can react further, thus forcing the equilibrium to completion [reaction (2)]:

\[
\text{base'} + \cdot\text{C—H} \rightleftharpoons \text{base—H} + \cdot\text{C}
\]

\[
\cdot\text{C} \rightarrow \text{break-down products.}
\]

It is unlikely that the direct mechanism is of much importance for fluid aqueous DNA, but it may be of considerable importance for DNA in chromatin for two reasons: (i) this is so highly organised and tightly packaged that there is not much room for water so that water damage becomes much less important; (ii) nuclear water comprises concentrated solutions of a wide range of organic compounds all of which are reactive towards water radicals. Hence the only radicals likely to attack DNA are those generated close to DNA. This greatly limits the effective aqueous target volume, thereby again reducing the probability of indirect damage. Therefore, direct damage needs to be considered. Our approach, following that of Gregoli and co-workers,5-7 has been to use frozen aqueous solutions of DNA.8-11 Others have used either dry DNA or partially hydrated DNA, and in particular, Gräslund, Hüttermann and their co-workers12 have used stretched DNA ribbon, which is largely oriented along the long axis, in order to obtain better EPR information than can be obtained from the frozen solutions.

The frozen solutions that we have used have the advantage that the DNA is fully solvated. Also, it is easy to incorporate additives designed to modify the course of DNA damage. On
freezing, pure ice crystals grow out until a 'glassy' DNA phase solidifies. Additives are totally rejected by the ice, and usually remain in the DNA phase and hence are close to the DNA. This convenient system breaks down if a third phase, comprising pure or hydrated additive, also crystallises out.

Dimethyl sulphoxide (DMSO) is of great importance as an additive to cellular systems, since it acts as a cryoprotector. It is also found to be a radioprotector in that it suppresses radiation-induced cell transformation in vitro.1,2 It is widely used as an OH radical scavenger in radiation-chemical studies, since it reacts at near diffusion rates to give the sulphuranyl radical adduct (I), which readily loses a methyl radical.4,5 It is used extensively in biological radiation studies specifically to scavenge OH radicals.6-8 A disadvantage is that the generation of reactive methyl radicals, and, of course, the need for relatively high concentrations if efficient competition is to be achieved.

DMSO reacts slowly with solvated electrons (k = 1.7 x 10⁶ mol⁻¹ s⁻¹), but in the pure solid, reaction with ejected electrons occurs to give methyl radicals, the expected radical-anions being apparently too unstable to be detected.9,10

\[
\text{Me}_2\text{SO} + e^- \rightarrow [\text{Me}_2\text{SO}^-] \rightarrow \text{Me}^- + \text{MeSO}^-
\] (3)

Electron loss gives the radical cation. This species has been prepared unambiguously in CFCl₃ at 77 K and has been characterised by a septet EPR spectrum and large g-value variation.12 In pulse radiolysis studies a transient with λₘₚ = 600 nm was identified as the parent radical cation, or as a breakdown product therefrom.13 In the present work we have found that the parentcation formed in CFCl₃ at 77 K has an intense violet colour, indicating an absorption band close to 600 nm. Hence we are able to confirm that the species detected in the liquid state was indeed the parent cation.

t-Butyl alcohol is also favoured as an OH radical scavenger by radiation chemists.23,24 Its reaction with solvated electrons is unimportant, but it reacts very rapidly to give \( \text{CH}_3\text{-C(Me}_2\text{)}\text{OH} \) with OH radicals. In the solid-state it reacts with 'dry' electrons to give t-butyl radicals,

\[
\text{(CH}_3\text{)}_3\text{COH} + e^- \rightarrow (\text{CH}_3\text{)}_3\text{C}^- + \text{OH}^-
\] (4)

and the radical cations deprotonate to give H₂C(C(Me₂)OH radicals, or lose CH₃ to give acetone.25

\[
\begin{align*}
\text{(CH}_3\text{)}_2\text{COH}^+ & \rightarrow (\text{CH}_3\text{)}_2\text{CO}^- + \text{CH}_3 + (\text{CH}_3\text{)}_2\text{CO}^- \\
& \rightarrow \text{H}_2\text{C(}\text{CH}_3\text{)}_2\text{OH}
\end{align*}
\] (5)

In our previous study of the action of hydrogen peroxide on DNA,11 no significant changes were detected in the absence of radiation, but on exposure to ⁶⁰⁰ Co γ-rays at 77 K the yields of DNA radicals were reduced and HO₂ radicals, together with species identified as sugar radicals, were detected.11 In the present work we have used UV light to generate OH radicals exclusively, thereby avoiding the competing generation of DNA radicals by the direct mechanism. This was one of the first techniques used to generate matrix isolated radicals for EPR study,26,27 and remains a powerful method for studying reactions of OH radicals.

Results and Discussion

DNA + Hydrogen Peroxide

Using light from a high-pressure mercury lamp (mainly 313 + 365 nm) there was no significant DNA damage in the absence of hydrogen peroxide. However, in its presence, spectra in the free-spin region grew in (fig. 2), which at high microwave powers showed a low-field doublet characteristic of HO₂ radicals (b). The central features comprise a broad singlet together with a poorly defined 15 G sextet. Despite the liquid-phase results, which strongly indicate preferential addition of OH radicals to the bases of DNA, our results seem to favour H-atom abstraction from the deoxyribose units for solid systems at low temperatures. Thus, EPR spectra for most possible OH radical DNA base adducts have been reported (table 1), but we have found no evidence for any of these adducts, which are all expected to be stable at 77 K despite their rapid reactions at room temperature. The only reasonable candidate is the thymine OH adduct for which we obtained a 20 G quintet in the solid-state (table 1). This differs sufficiently from the 15 G features of fig. 2(a) that we consider that it cannot be a major product. The sextet may, in part, be due to C₅ centred radicals (II). This radical has no α-protons, so relatively narrow lines are expected.

\[
\begin{align*}
\text{O} & \quad \text{CH₂} \\
\text{PO} & \quad \text{H} \\
\text{B} & \quad \text{H}
\end{align*}
\] (II)

Fig. 2. First derivative X-band EPR spectra for a hydrogen peroxide-DNA solution photolyzed at 77 K, (a) at low microwave power, showing features assigned to a sugar radical (six lines) + a central singlet, and (b) at high power, showing features assigned to HO₂ radicals.
### Table 1. Radicals formed from DNA bases and deoxyribose units by 'OH radical attack

<table>
<thead>
<tr>
<th>Radical</th>
<th>Hyperfine coupling/G*</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>(TOH)</td>
<td></td>
<td>3H ≈ 20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1H ≈ 20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Quintet (20 G) expected</td>
</tr>
<tr>
<td>(COH)</td>
<td>(1H ≈ 20)</td>
<td>Triplet (20 G) expected</td>
</tr>
<tr>
<td>('GOH or 'AOH)</td>
<td></td>
<td>('GOH)↑↑N(↑) ≈ 21 G</td>
</tr>
<tr>
<td></td>
<td></td>
<td>* Major doublet with extra</td>
</tr>
<tr>
<td></td>
<td></td>
<td>parallel features</td>
</tr>
<tr>
<td></td>
<td></td>
<td>('AOH is expected to be similar)</td>
</tr>
<tr>
<td>('C₁)</td>
<td></td>
<td>1H ≈ 23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1H ≈ 45</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Quartet (∼ 23 G) expected</td>
</tr>
<tr>
<td>('C₂)</td>
<td>1H ≈ 22</td>
<td>1H ≈ 48</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1H ≈ 6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Quartet (∼ 25 G) expected</td>
</tr>
<tr>
<td>('C₃)</td>
<td></td>
<td>1H ≈ 13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1H ≈ 45</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1H ≈ 15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sextet (∼ 15 G) expected</td>
</tr>
<tr>
<td>('C₄)</td>
<td></td>
<td>1H ≈ 15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1H ≈ 33</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1H ≈ 8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Quartet (∼ 16 G) expected</td>
</tr>
<tr>
<td>('C₅)</td>
<td>1H ≈ 20</td>
<td>1H ≈ 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Doublet (20 G) expected</td>
</tr>
</tbody>
</table>

* 1 G = 10⁻¹⁴ T; * data in parentheses are expectation values only; * ref. (32); * ref. (30); * ref. (33); * ref. (34).

---

A stick diagram, using the data given in table 1 (included in fig. 2) shows that this assignment is reasonable, but we do not consider that it is fully established. Also, there may be other anisotropic features present from other sugar radicals which are largely concealed by the sextet and singlet. It is, of course, difficult to identify the broad singlet which is always present in the spectra. None of the radicals discussed above is expected to give such a spectrum. There is a contribution from the 'perpendicular' features for HO₂ radicals [fig. 2(c)] but another centre is also present. Guanine cations (G⁺) give rise to a very similar singlet (same average g value and same width), and we tentatively suggest that these cations may be formed from 'OH radicals under our conditions. (They are not formed on photolysis in the absence of H₂O₂.) Guanine has a low ionization potential (8.24 eV), and 'OH radicals have a high electron affinity. If some 'OH radicals are formed close enough to G for electron-transfer to be reasonable (<20 Å), but too far away from any reactive unit to permit direct attack, then the following reaction may occur by electron tunnelling

\[
G + 'OH \rightarrow G^+ + OH^-. \tag{6}
\]

At present, we have no other suggestions for the structure of the species responsible for this central feature. Absence of high yields of 'OH radical base-adducts probably reflects the inaccessibility of the bases at 77 K. The most mobile species under these conditions are electrons, which can travel considerable distances along the DNA spine. Protons can also migrate, but 'OH radicals are likely to be trapped or to react with their near neighbours. There is no clear evidence for trapped 'OH radicals, but we stress that, in glassy media, their EPR features are not well defined. We suggest that they react with their near neighbours, which are expected to be H₂O₂ molecules (giving HO₂) and the outer regions of the DNA, which are mainly deoxyribose units.

One of the most exposed C—H groups is C₅—H, and this may explain why the 'C₅ radical appears to be of importance in this study. The absence of this centre in the radiolysis of...
DNA reinforces our conclusion that 'OH radical attack is not of major importance under these conditions.

DNA–DMSO System

In our studies of electron-donor and -acceptor additives, there have been clear reductions in the yields of the major DNA damage centres (G'4 and T"/C") for additive : base-pair ratios of ca. 1 : 50. However, for DMSO systems, even at ratios of 1 : 1 the [G']4 and [T"/C"] are almost unchanged. (Although we and others have previously assumed that the major e"-capture centre is T", there is growing evidence that C" is also important.) This result also supports the contention that 'OH radical reactions are not an important source of DNA damage under our conditions.

However, there are interesting effects for higher [DMSO]. Increasing the DMSO : base-pair ratio to ca. 10 : 1 results in a clear loss of DNA radicals, and the appearance of signals from CH3 and H2CSO(CH3) radicals (fig. 3). The EPR resembles that obtained from concentrated aqueous DMSO solutions after exposure at 77 K, but the large reduction in [DNA'] radicals means that these do not simply arise as a result of damage to a separate H2O-DMSO phase. Since the ionization potential of DMSO (9.1 eV) is greater than that of G" (8.24 eV) hole transfer should not favour DMSO damage.

The problem is, not the appearance of DMSO radicals, since these must surely be formed at high concentrations of DMSO, but the decrease in DNA radicals, when DMSO is so ineffective at low concentrations [fig. 3(b)]. Probably several factors contribute: (i) the decrease in [H2O] in the DNA zone may facilitate electron return thus decreasing [G']4 and [T"/C"], (ii) the reaction of H2O to give, ultimately, G", may become less favourable than reaction with DMSO to give DMSO"; (iii) DMSO molecules may intercept electrons before they reach T or C or before these units have time to relax to form protonated units. Also, electron-transfer between DMSO molecules may help to remove e" from the vicinity of DNA.

DNA—e-Butyl Alcohol

As with DMSO, at relative concentrations up to ca. 1 : 1 (TBA : base-pairs) there is almost no effect on the concentration of DNA radicals or their behaviour on annealing. Despite the relatively high concentration of TBA, there is no detectable yield of TBA radicals. Even CH3 and (CH3)3C, which can be detected in very low concentrations because of their narrow features even at 77 K, were not detectable.

This result accords with those from the DMSO and hydrogen peroxide studies, showing that, in marked contrast to the liquid-phase, attack by 'OH radicals formed by γ-radiolysis, must be insignificant.

Results at higher [TBA] contrast significantly with those for DMSO. Yields of TBA radicals [CH3, (CH3)3C and H2CSO(CH3)] remain very low, but yields of DNA radicals (G" , T"/C" and TH) increase, rather than decrease (fig. 4). Thus TBA appears to act as a radiosensitiser, whereas DMSO acts as a poor radioprotector.

This curious contrast can be understood in the following way. In both cases, the aquated cosolvent molecules are forced into the vicinity of the DNA, thereby potentially increasing the DNA target volume. (This volume effect is well defined for inert salt systems, and will be elaborated separately.) In the case of TBA, electron-capture [eqn (4)] and hole-capture [eqn (5)] are clearly very inefficient, so that both electrons and holes are mobile and can reach the DNA, thereby increasing the yields of DNA radicals.

However, for DMSO, the rates of both e" and hole capture are greater and can compete even with capture by DNA so that DMSO radicals dominate at high concentrations.

Strand-breaking Studies

In most of our previous work we have correlated the EPR data with measurements of strand breaks after annealing to room temperature. In the present work, only TBA systems have so far been studied. We note that, in this case, TBA in the low concentration range has very little effect on the number of strand-breaks, in accord with the EPR results, whilst in fluid solution marked protection was observed.

We thank the Cancer Research Campaign (CRC), Ministry of Defence (MOD) and Association for International Cancer Research (AICR).
Fig. 4. (a) First derivative X-band EPR spectrum for a TBA-DNA (20:1) solution after exposure to $^{60}$Co $\gamma$-rays at 77 K and annealing to ca. 208 K, showing features primarily assigned to TH radicals. The intensity of these features is ca. twice that in the absence of TBA. (b) Effect of [TBA] on the intensities of DNA radical signals (C* -f T -/C ) at 150 K (-----) and of TH radical signals at 170 K ( ).

References

41 B. W. Wren, Ph.D. Thesis (Leicester University, 1985).